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ANTIBODY RESPONSE AFTER IMMUNOTRANSFUSION IN MALIGNANT ENDOCARDITIS

KATHARINE M. HOWELL, BERNARD PORTIS
AND
DOROTHY A. BEVERLEY

From the Albert Kuppenheimer Fund of the Michael Reese Hospital and the Nelson Morris Memorial Institute for Medical Research, Chicago

During the last 10 years transfusion of normal or immune blood has been used frequently as a therapeutic measure in streptococcus endocarditis, chronic streptococcus wound infections, and in septicemia due to various bacteria.

In 1919 Wright,[1] in his "Lecture on the Lessons of the War," stated that simple transfusion had been used extensively many times without marked success. The failure of simple transfusion was due, he thought, to 2 factors: (1) That the normal blood transfused was actually a good culture medium for the bacteria in the patient's blood stream; (2) that the transfused leukocytes did not do so effective bactericidal work in the rapidly flowing blood stream as they did outside the blood. He also tried immuno-transfusion, with which the results, particularly in streptococcus infection, were more favorable, and worthy, he thought, of further trial. Wright, Colebrook, and Storer,[2] and Colebrook and Storer[3] extended the original observations of Wright on immuno-transfusion, and they came to the conclusion that it was advantageous, particularly in streptococcus infections. Many physicians have reported their experience with simple and immune transfusions. The diversity of results may be illustrated by the conclusions of the following investigators. McClure and Dunn,[4] successfully used a vaccinating transfusion in a case of typhoid fever, and they considered that transfusions of such nature should be tried in other diseases. Hooker[5] reported good results in the treatment of staphylococcus septicemia by transfusion of immunized blood. Little[6] believed that an immunized blood transfusion saved the life of a patient with epidemic influenza (mixed infection) and local abscesses. Robertson[7] reported a successful result in a case of endocarditis, with a positive streptococcus viridans blood culture; he used six transfusions from an immunized donor in doses varying from 200 to 300 cc. of whole blood. Fry[8] used whole blood transfusions from immunized donors in the treatment of pyogenic infections, and he considered that the transfusions were a help, particularly in the chronic suppurative infections. Libman and Ottenberg[9] immunized donors, for use in transfusion, against the particular organism isolated from bacterial endocarditis. Contrary to the experience of Wright[1] they did not find immunized transfusion superior to simple transfusion. In subacute streptococcus endocarditis they found transfusion valuable as a supporting measure and they noted that the temporary results were sometimes brilliant. Lamb[10] reported that in his experience immunized transfusion was an important therapeutic measure, modifying the symptoms in endocarditis although not affecting a cure. Kinsella,[11] on the other hand, reported that no noticeable benefit occurred after transfusion in the cases of subacute

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streptococcus endocarditis under his observation. Dick[12] considered that antistreptococcus whole blood produced a definite temporary improvement in malignant endocarditis. Unger[13] reported that 2 patients with subacute bacterial endocarditis transfused with blood from healthy donors died, while 5 out of 7 patients treated with immunized blood recovered.

From these reports and others, transfusion of immunized blood seems worthy at least of trial in cases of chronic infection, in septicemia, and in streptococcic endocarditis. It seems to be indicated that transfusion should be given a thorough trial in streptococcic endocarditis, since autogenous vaccines and antistreptococcus serum have been found by Lamb,[10] Weaver,[14] Hudson,[15] McCloud,[16] and many other investigators to be of little or no value in this infection.

Healthy young men are chosen for immunization, and their blood must belong to an iso-agglutinin group compatible with that of the recipient. It is also profitable to test the leukocytic activity of the donor's blood. The method used by most investigators for immunization is to inject the donors with repeated doses of a vaccine prepared from the bacterium isolated from the recipient's blood or from an infected lesion. After the donor is immunized, he is bled and the whole blood or the citrated blood or the defibrinated blood, or in some instances, the serum only, according to the investigator's method, is given intravenously to the patient. Other methods of immunization have been described. For example, Wright, Colebrook, and Storer,[1, 2, 3] have experimented with the following methods: injecting blood plus a vaccine simultaneously; injecting a vaccine intravenously into the patient just before he is given a transfusion; injecting a vaccine subcutaneously in the donor, 4 or 5 hours before his blood is withdrawn for transfusion.

While bacterial endocarditis is usually benefited, temporarily at least, by the transfusion of immune blood, the exact mechanism by which this is accomplished is not understood. The following factors have been suggested. Some investigators think that benefit is derived entirely from the simple replacement of damaged red cells by new ones, thereby reducing the patient's anemia and indirectly his other symptoms; also by replacing the damaged leukocytes by active leukocytes. It has been suggested by Fry[8] that the improvement might be due to protein shock. If the above factors only are involved, the immune transfusion should have no advantage over simple transfusion. In addition to these factors it seems probable that the antibactericidal mass action of the immune blood is an important factor in the benefit derived from immuno-transfusion.

The following report of a case of acute bacterial endocarditis treated with immunized blood is considered worthy of presentation for the following reasons: the bacteriology was of unusual interest; the antibody titer of the patient and his donors was followed over the 5 month period of treatment; the possibilities and limitations of the therapeutic value of immune transfusion in cases of acute bacterial endocarditis were demonstrated.

BACTERIOLOGY

Three cultures of streptococcus were obtained on separate occasions from the blood of a patient with clinical symptoms of malignant bacterial endocarditis. The growth of each of the positive blood cultures appeared as a fine, granular, nonhemolytic streak on blood-agar slants. On blood-agar poured plates examined after 24 hours 2 types of colonies appeared:

(1) small, moist, elevated, dark green colonies, and (2) small, moist, elevated colonies surrounded by a narrow zone of hemolysis. In films made from blood-agar slants and from broth cultures, were seen fine, gram-positive, long chained streptococci and large, gram-positive cocci occurring in pairs and short chains. Films made from individual colonies on the blood-agar poured plates indicated that the fine, long chained streptococcus was always obtained from hemolytic colonies and that the large, short chained streptococcus was always obtained from the green colonies. Neither type of streptococcus was bile soluble and according to Holman's [17] classification the hemolytic streptococcus was Streptococcus infrequens and the viridans was Streptococcus mitis.

The first cultures contained a majority of the fine cocci, but after several subcultures the larger coccus predominated. The second culture, which was obtained 2 months after the first, had a predominence of the larger coccus from the first. The patient's agglutinin titer for the large coccus at this time was 1 : 20, and the agglutinin titer for the smaller coccus was 1 : 2560. The third culture, which was obtained several weeks after the second, contained the 2 organisms in about equal proportion. The fourth blood culture, taken a month later, when the patient's agglutinin titer for the large coccus was 1 : 1280 and for the small coccus 1 : 10,240, did not grow. Apparently the blood stream was sterilized at this period, a finding that is the reverse of Lamb [10] and others.

IMMUNIZATION OF DONORS

A vaccine was prepared from the first subculture of the streptococci obtained from the patient's blood, the one that contained a predominence of the smaller coccus. Five healthy young men were selected for donors. The patient's blood belonged to group 2; the donors, therefore, were chosen from group 2; the patient's blood was compatible with that of each donor, and the donors were compatible with each other. In addition, a direct compatibility test was done just before each transfusion. The donors were immunized by repeated injections, at 5 day intervals, of the streptococcus vaccine. Immunization as measured by agglutinin titer, was obtained within 2 weeks of the initial vaccination. When the 2 types of streptococcus were found to be morphologically and culturally distinct types, a second vaccine was prepared that contained a predominence of the larger organism and an equal dose of each vaccine was used in immunization until later vaccines could be prepared separately from the streptococcus viridans and from the streptococcus hemolyticus. The

dose of each vaccine then was measured as the agglutinin titer for each separate organism indicated.

The immune blood was given by direct transfusion in 200 cc. to 300 cc. amounts.

RESULTS OF TRANSFUSION WITH IMMUNE BLOOD

Twelve transfusions of blood from donors immunized with the streptococci that had been isolated from the patient's blood were given. The 12 transfusions extended over a period of 5 months. Before the patient received immune transfusion his serum contained no agglutinin for the streptococci isolated. The opsonic index was 0.57 and the complement-fixation test was weakly positive.

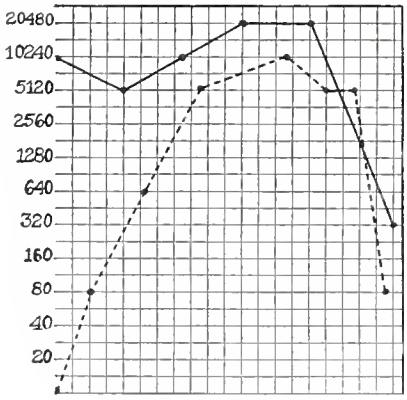

Chart 1.—Agglutinin response to mixed streptococcus vaccine. 1 sq. on abscissa = 2 days. —— donor. - - - - recipient.

Agglutinins.—Transfusion 1. The recipient received 200 cc. of blood than had an agglutinin titer of 1 : 10,240. Three days after the transfusion the recipient's titer was 1 : 80.

Transfusion 2. The recipient received 200 cc. of blood that had a titer of 1 : 5120. The recipient's titer rose to 1 : 640.

Transfusion 3. The recipient received 200 cc. of blood that had an agglutinin titer for streptococci of 1 : 10,240. The recipient's titer rose to 1 : 5120.

Transfusion 4. The recipient received 200 cc. of blood that had an agglutinin titer of 1 : 20,480. The recipient's titer rose to 1 : 10,240.

Transfusion 5. The recipient received 200 cc. of blood with an agglutinin titer of 1 : 10,240. The recipient's titer after this transfusion was 1 : 7000. This titer persisted for 3 days and then suddenly dropped to 1 : 80.

Transfusion 6. The recipient received 300 cc. of blood with an agglutinin titer of 1 : 320.

These transfusions had been given at about 2-week intervals. At this time it was decided to measure the agglutinin titer of the recipient's serum and the donor's serum for the 2 apparently different types of coccus. (Charts 2 and 3.) The recipient's titer for the streptococcus viridans was 1 : 20 and for the streptococcus hemolyticus 1 : 2560.

Transfusion 7. The recipient received 300 cc. of blood from a fresh donor whose agglutinin titer for streptococcus viridans was 0 and whose

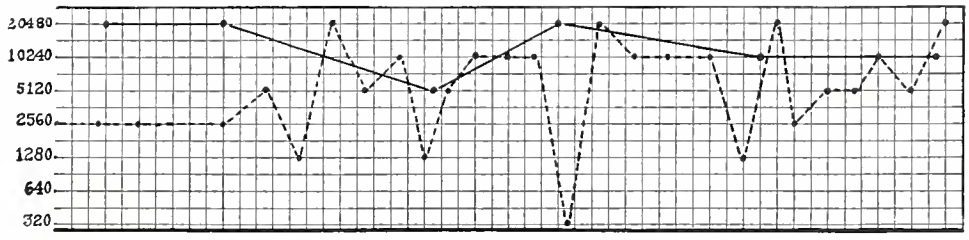

Chart 2.—Agglutinin response to hemolytic streptococcus vaccine. 1 sq. on abscissa = 2 days. —— donor. - - - - recipient.

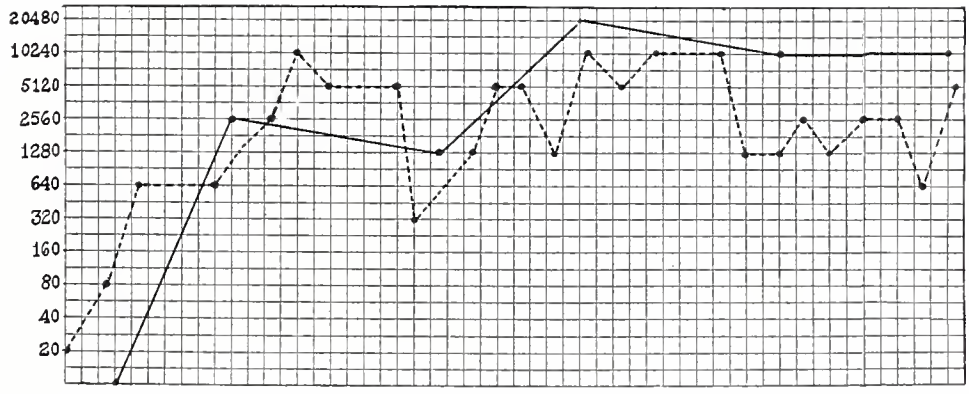

Chart 3.—Agglutinin response to streptococcus viridans vaccine. 1 sq. on abscissa = 2 days. —— donor. - - - - recipient.

titer for the streptococcus hemolyticus was 1 : 20,480. The recipient's titer rose to 1 : 640 for the streptococcus viridans organism in spite of the fact that the immune serum received had contained no agglutinin for this coccus, and that his previous titer for this organism was only 1 : 20. For the streptococcus hemolyticus the titer remained 1 : 2560.

Transfusion 8. The recipient received 300 cc. of blood from a fresh donor that had a titer for the streptococcus viridans of 1 : 2560 and a titer for the streptococcus hemolyticus of 1 : 20,480. The recipient's

agglutinin titer increased to 1 : 10,240 for the streptococcus viridans, and to 1 : 20,480 for the streptococcus hemolyticus. It is of interest to note that the titer for the streptococcus viridans was again much higher than the titer of the blood received in transfusion. The recipient's agglutinin titer remained high for a period of 3 weeks; then there was an abrupt drop to 1 : 320 for the streptococcus viridans and 1 : 1280 for the streptococcus hemolyticus.

Transfusion 9. The recipient received 300 cc. of blood that had a titer of 1 : 1280 for streptococcus viridans and 1 : 5120 for streptococcus hemolyticus. The recipient's titer increased to 1 : 5120 for the streptococcus viridans and 1 : 10,240 for the streptococcus hemolyticus. In both instances, this was a higher titer than that of the serum received; however, there was another abrupt drop after 2½ weeks to 1 : 1280 for the streptococcus viridans and 1 : 320 for the streptococcus hemolyticus.

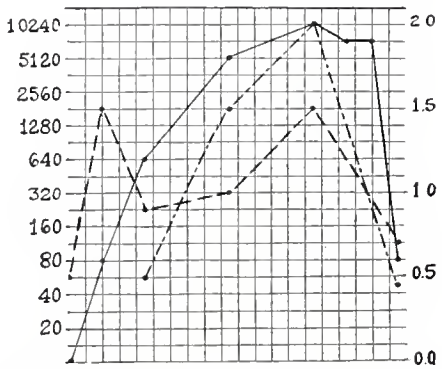

Chart 4.—Agglutinin and opsonin response to mixed streptococcus vaccine. 2 sq. = 1 day. Agglutinin titer on left. Opsonic index on right. —— recipient's agglutinin. —— - —— - —— recipient's opsonin. - - - - donor's opsonin.

Transfusion 10. The recipient received 300 cc. of blood that had a titer of 1 : 20,480 for each type of streptococcus. The recipient's titer increased to 1 : 10,240 for the streptococcus viridans and 1 : 20,480 for the streptococcus hemolyticus. After 2½ weeks the titer for each organism had dropped to 1 : 1280.

Transfusion 11.—The recipient received 300 cc. of blood with a titer of 1 : 10,240 for each type of streptococcus. The recipient's titer 3 days after this transfusion was 1 : 1280 for the streptococcus viridans and 1 : 20,480 for the streptococcus hemolyticus. The titer remained high for 3 weeks; at that time the titer for streptococcus viridans dropped to 1 : 640 and for the hemolytic organism 1 : 5120.

Transfusion 12. The recipient received 300 cc. of blood that had an agglutinin titer of 1 : 10,240 for each type of streptococcus. The

recipient's serum 3 days after this transfusion had a titer of 1 : 5120 for the streptococcus viridans and a titer of 1 :20,480 for the streptococcus hemolyticus.

Transfusions and observations on agglutinin titer were discontinued at this time. In these 12 observations the recipient's agglutinin titer for streptococcus viridans on 8 occasions was higher than that of the blood received and in 7 instances the recipient's titer for streptococcus hemolyticus was higher than that of the blood received in the preceding transfusion; apparently, therefore, there was active immunization in addition to passive immunization of the recipient.

Opsonin.—The opsonic index of each serum was determined at the same time that the agglutinin titer was measured. The curves of the

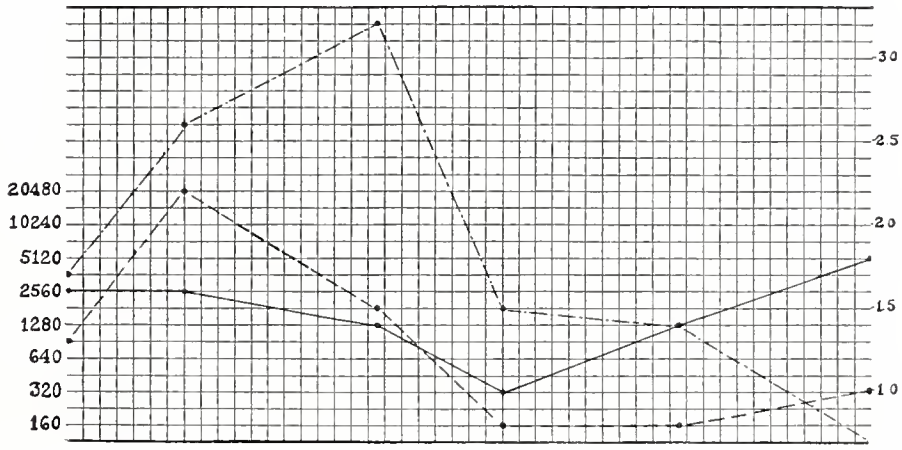

Chart 5.—Agglutinin and opsonin response to hemolytic streptococcus vaccine. 1 sq. = 2 days. Agglutinin titer on left. Opsonic index on right. —— recipient's agglutinin. —— - —— - —— recipient's opsonin. - - - - donor's opsonin.

opsonic indices of the recipient's and of the donor's serum closely paralleled those of the agglutinin curves (charts 4, 5 and 6). When the recipient's agglutinin titer dropped from 1 : 10,240 to 1 : 80, the opsonic index dropped from 2.0 to 0.42, and conversely when the agglutinin titer rose to 1 : 20,480 the opsonic index rose from 1.00 to 2.88. The recipient's initial opsonic index was 0.57, and after the first transfusion of immunized blood it rose to 1.50. His highest opsonic index for the combined streptococcus viridans and the streptococcus hemolyticus suspensions was 2.00; for the streptococcus viridans 2.70; for the streptococcus hemolyticus, 3.30. The final opsonic indices, obtained 3 days after the last transfusion, were 2.50 for the streptococcus viridans and 0.30 for the streptococcus hemolyticus. The initial opsonic indices of

the donors serum were uniformly low, 0.91, 1.50, 0.57, 0.42, and 0.28, and they never rose above 1.70. In spite of the low opsonic indices of the donor's serums, the opsonic index of the recipient's serum increased after each transfusion. Active immunization in addition to passive immunization of the recipient was even more apparent in the opsonic response than it had been in the agglutinin response. (Charts 4, 5, and 6).

Complement-fixation antibodies.—Complement-fixation antibodies were present in low dilution in the recipient's serum before he received the transfusions of immune blood. After transfusion the complement-fixation reaction increased from a 2 plus to a 3 plus reading. The recipient's serum did not at any time completely fix complement (4 plus). Streptococcus complement-fixation antibodies could not be detected in

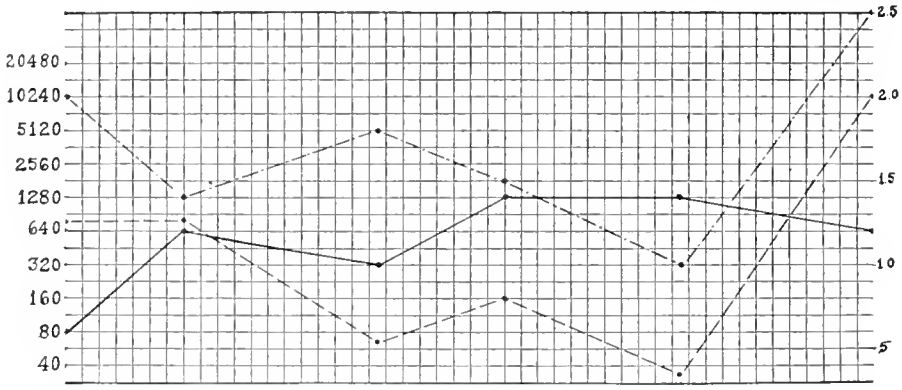

Chart 6.—Agglutinin and opsonin response to streptococcus viridans vaccine. 1 sq. = 2 days. Agglutinin titer on left. Opsonic index on right. —— recipient's agglutinin. —— · —— · —— recipient's opsonin. - - - - donor's opsonin.

the donor's serums before they received the antistreptococcus vaccine. These antibodies appeared in the serum later in the course of immunization than agglutinin and opsonin, but a 4 plus reaction was obtained in all serums during immunization. There was considerable fluctuation in the streptococcus fixation antibodies; for example, the readings on the recipient's serums were as follows: 2 plus, 3 plus, 3 plus, 1 plus, 3 plus, 0, 2 plus, 0. The streptococcus antibody response of the recipient's and the donors' serums was practically the same for the 3 streptococcus antigens, the combined streptococcus (viridans and hemolyticus), streptococcus hemolyticus, and streptococcus viridans. The complement-fixation antibody response in the recipient's serum differed from that of agglutinin and opsonin in that it did not rise so high in the recipient's serum as it did in the donor's serums.

DISCUSSION

The bacteriology in this case of acute bacterial endocarditis was of particular interest because it presented 2 possibilities: a mixed streptococcus infection; this is rather rare, although one other such case has been encountered by one of us [18] in which 2 distinct strains of streptococcus were isolated at necropsy from the same heart valve lesion; or a single streptococcus strain that was undergoing mutation. Mutation of streptococcus is a debated question in bacteriology at the present time and this streptococcus seemed to present an unusual opportunity for studying the possibility of mutation. The results of a detailed bacteriologic and serologic investigation of this organism will appear in a subsequent paper.

The patient's serum differed from that of most of the cases reported in which antibodies were measured (Wright,[1] Libman,[9] and others), in that the agglutinin and opsonin titers for the streptococcus isolated were lower before transfusion than those of normal serum, and the complement fixing antibodies were only very weakly positive. The active immunity response to the streptococcus infection of the patient, therefore, as measured by these 3 immunity tests was almost negative. However, as soon as the patient was transfused with immunized blood, there was undoubtedly active as well as passive immunization, because the agglutinin and opsonin titers were uniformly higher in the recipient's blood than they were in the transfused blood.

In spite of the fact that the recipient and the 5 donors were in the same iso-agglutinin blood group (2) and that a satisfactory direct compatibility test was always obtained before transfusion, the recipient had such severe systemic reactions after receiving the blood of donor 1 that the use of this donor's blood was discontinued after the second trial. With this exception the recipient's general symptoms were almost immediately improved. The improvement would last from 7 to 10 days, then his symptoms would become gradually worse, and there would be an accompanying drop in his antibody titer. A new transfusion would be followed by another temporary improvement. Although the recipient improved temporarily after each transfusion, he became progressively weaker and had showers of emboli with increasing frequency. He left the hospital after he had received immunizing transfusions for 5 months. He became rapidly worse and died about 1 week after the last transfusion. Immuno-transfusion probably prolonged for 4 or 5 months the life of this patient with acute bacterial endocarditis. At the time the

immuno-transfusions were started the patient's symptoms were so severe that death was expected momentarily. Since immuno-transfusion prolonged the life of the patient in this severe case of acute bacterial endocarditis, it seems possible that in subacute bacterial endocarditis it might effect a cure.

SUMMARY

In a case of acute malignant endocarditis, both Streptococcus hemolyticus and Streptococcus viridans were isolated from the blood on 3 different occasions.

During a period of 5 months the patient received 12 transfusions of immune whole blood.

Before immuno-transfusion was begun his blood serum contained no agglutinin for the streptococci isolated. The opsonic index was 0.57 and the complement-fixation reaction with streptococcus antigen was weakly positive.

The serum of the donors at the time of transfusion had an agglutinin titer of 1 : 10,240 or 1 : 20,480.

The agglutinin titer of the recipient's serum increased after each transfusion, reaching after the later ones a figure higher than that of the transfused blood.

The opsonic index of the recipient's blood serum also increased after each transfusion, as a rule running parallel with the agglutinin titer, and reaching values higher than those of the transfused blood.

Complement-fixing antibodies also increased, but appeared later than agglutinin and opsonin and fluctuated more irregularly.

The increase in specific antibodies of the recipient's blood, both as compared with its own original content and with that of the immune donors, is interpreted as evidence of the development of active as well as passive immunity as the result of immuno-transfusion.

At variable periods, usually 2 to 3 weeks, after each transfusion the agglutinin and opsonin titers of the patient's blood dropped suddenly to low figures, reaching a maximum again usually within 12 days after the next transfusion.

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APPARENT MUTATION OF STREPTOCOCCI FROM ACUTE MALIGNANT ENDOCARDITIS

KATHARINE M. HOWELL AND DOROTHY A. BEVERLEY

From the Albert Kuppenheimer Fund of the Michael Reese Hospital and the Nelson Morris Memorial Institute for Medical Research, Chicago

In the clinical report [1] of a patient with acute malignant endocarditis treated by immuno-transfusion, a brief description was given of the streptococcus isolated from the blood cultures. This streptococcus appeared of unusual interest, and its bacteriologic and serologic reactions have been observed over a period of 2 years.

BACTERIOLOGY

Positive blood cultures obtained on 3 separate occasions contained upon microscopic examination gram-positive cocci differing in morphology and arrangement. When films from the first culture were found to contain 2 types of coccus the question arose whether we were dealing with a contaminated culture, with a rapidly growing culture containing involuted forms, with 2 distinct organisms, or with a single strain undergoing mutation. Ascites dextrose broth cultures of the organisms grew luxuriantly. On blood-agar slants there was a scanty, finely granular growth composed of minute, slightly elevated, moist colonies. On streaked blood-agar plates there was no hemolysis nor brown nor green coloration seen on 24-hour plates. After 48 to 72 hours, however, the streaks sometimes appeared slightly hemolytic or green when the plates were held to the light. Although the minute colonies appeared identical, stained films from the streak growth as well as from the broth cultures contained 2 distinct types of organisms. One was a very small, round, gram-positive coccus, occurring in chains of 10, 12, or more. The other variety was a gram-positive, lancet-shaped diplococcus, frequently occurring in chains of 6 or 8. The latter resembled a pneumococcus in morphology rather than a streptococcus, but broth cultures were not in any degree bile soluble, nor were the cocci agglutinated by antipneumococcus serums. Poured blood-agar plates were inoculated from the broth cultures. In 24 hours many fine, dark green colonies appeared on the plates and an occasional colony with a

Received for publication, March 13, 1926.

[1] Howell, K. M.; Portis, B., and Beverley, D. A.: J. Infect. Dis., 1926, 39, p. 1.

narrow zone of hemolysis. After 48 to 72 hours a zone of hemolysis also appeared about the green colonies. Both green and hemolytic colonies were picked and subcultured in ascites dextrose broth. In every instance green colonies gave rise to the large, lancet-shaped diplococcus, and the hemolytic colonies to the fine, long chained coccus. Replated, the lancet-shaped coccus always produced green colonies, and the fine coccus gave rise to hemolytic colonies. In broth cultures, the larger coccus from the green colonies gave a diffusely cloudy growth. The broth cultures from the hemolytic colonies gave a more granular growth. The latter had to be cultured every 24 hours because so much acid was formed that the coccus was killed. The sugar reactions of the fine, long chained, hemolytic organism were as follows: dextrose, dextrin, galactose, trehalose, mannit, saccharose, salicin, lactose, maltose, and levulose were fermented; dulcitol, xylose, inulin, adonite, and glycerol were not fermented. The viridans streptococcus fermented dextrose, dextrin, galactose, trehalose, salicin, lactose, maltose and levulose; it did not ferment dulcitol, mannit, xylose, inulin, adonite, glycerol and saccharose. According to Holman's classification based on the fermentation of dextrose, lactose, mannit, and salicin, the fine, long chained streptococcus was Streptococcus infrequens, and the green-producing streptococcus was Streptococcus mitis.

In the first blood-agar culture, A, obtained from the patient, the hemolytic streptococcus predominated, but in subcultures there was an increase and final predominance of the green-producing streptococcus. A second blood culture, B, obtained 2 months after the first positive culture, had a predominance of the streptococcus viridans from the first (the patient's agglutinin titer for the green-producing streptococcus at this time was 1:20 and for the hemolytic streptococcus 1:2560). The third culture, C, obtained 2 weeks after the second, contained the 2 organisms in about equal proportion.

Many poured blood-agar plates were made from the third positive blood culture. The majority of the colonies that appeared within 24 hours were green; but there was an occasional hemolytic colony, and a large number of small, moist, slightly elevated colonies that were neither green nor hemolytic. Films from these anhemolytic colonies showed streptococci similar in appearance to those from hemolytic colonies. The green-producing and the hemolytic streptococci were morphologically and culturally identical with those isolated from the first and second blood cultures. The anhemolytic colonies were identical in their cultural reaction to the hemolytic colonies. When the anhemolytic strain

was alternated daily from blood agar to ascites broth and back to agar, etc., the anhemolytic character of the streptococcus remained constant.

The fact that the second and third blood culture contained the 2 morphologically and culturally distinct streptococci ruled out the possibility that there was a contaminant in the first blood culture. The hemolytic and nonhemolytic colonies ruled out the possibility that we had a rapidly growing strain containing involuted forms. Attention was concentrated, therefore, on whether we had 2 or even 3 distinct strains of streptococci or whether we had 1 strain that was undergoing mutation.

Mutation.—The reports of various investigators indicate that there is mutation in the streptococcus group of bacteria, both in vitro and in vivo.

Dobell's [2] definition that mutation is a permanent change which takes place in a bacterium and is then transmitted to subsequent generations should be followed. Ashner [3] reported that there were many variations in the streptococcus group (71 organisms) due to changes in the mediums, but that they were of a temporary nature. He noted a few permanent changes, such as loss of capsule in mucosus capsulatus, a loss of the power to ferment inulin, and a few changes in the hemolyzing quality. He believed that it was essential to work with a pure line from a single organism in order to consider an apparent change true mutation. Broadhurst [4] claimed that the fermentation response of a large percentage of streptococci was consistent if identical cultural conditions were maintained. Cole and Wright [5] considered that mutation occurred spontaneously. They, too, held that it was necessary to work experimentally with a pure line from a single bacterium for the following reasons: a colony might contain 2 strains, and under varying conditions, one or the other strain might predominate in such culture; one strain might be suppressed for a while and then under changing conditions reappear; or one strain might die. All three of these conditions would give the impression of mutation. They considered mutation due to brief animal passage unreliable, because of the many chances for the appearance of contaminating strains. Browning and Gulbransen [6] noted that there were marked alterations in morphology and in biological characteristics of bacteria belonging to the streptococcus group. Bordet [7] reported that a streptococcus under his observation developed a capsule in vivo and that this development of capsule was accompanied by increased virulence. Rosenow [8] reported changes in the streptococcus and pneumococcus groups. Clawson [9] reported that typical hemolytic streptococcus strains, after they were cultured on artificial media from 6 months to 3 years, would sometimes split off green-producing variants. Valentine [10] reported a similar experience. Kuczynski and Wolf [11] reported that in their experience a freshly isolated strain of Streptococcus viridans would split off a progressive

[2] J. Genetics, 1912, 2, p. 325.
[3] J. Infect. Dis., 1917, 21, p. 409.
[4] Science, 1914, 39, p. 798.
[5] J. Infect. Dis., 1916, 19, p. 209.
[6] J. Path. and Bact., 1923, 26, p. 121.
[7] Tráité de l'immunité dans les Maladies infectieuses, 1920.
[8] J. Infect. Dis., 1914, 14, p. 1.
[9] J. Infect. Dis., 1920, 27, p. 368.
[10] J. Exper. Med., 1917, 36, p. 157.
[11] Ztschr. f. Hyg. u. Infektionskrankh., 1921, 92, p. 119.

number of hemolytic colonies until all the colonies as shown by blood-agar plate would be hemolytic. Schnitzer and Münter [12] reported that by brief animal passage they could change hemolytic streptococci into viridans, and reverse regardless of whether the strains were virulent or avirulent, freshly isolated or stock cultures. Hintze and Kühne [13] considered that in certain instances hemolytic streptococci might be changed into green-producing streptococci by a brief sojourn (2 to 4 hours) in the peritoneal cavity of a mouse, or that mutation might take place after prolonged cultivation of many generations on artificial culture media. Rolly,[14] on the contrary, reported that in his experiments he had no proof that hemolytic streptococcus was changed into nonhemolytic streptococcus in animal passage.

The following bacteriologic and serologic tests have been undertaken in an attempt to determine whether we had 2 or 3 distinct strains of streptococci or whether we had 1 strain that was undergoing mutation.

Bacteriologic observations on the 6 streptococci isolated from the 3 positive blood cultures were made over a period of 2 years in order to observe whether any mutation took place spontaneously on artificial culture medium. Weekly observations were made of these 6 streptococci in ascites, dextrose, veal infusion broth P_H 7.6, and on human blood-agar poured plates. The 4 diagnostic sugar fermentations were observed at monthly intervals.

For several weeks after isolation each streptococcus was subcultured daily, alternately between ascites dextrose broth and blood-agar plates. Occasionally the colonies suggested mutation. For example, 5 weeks after isolation, 6 ascites dextrose broth subcultures were made from a blood-agar streak culture of Streptococcus hemolyticus, B. The microscopic examination of the broth culture showed fine, long chained cocci in 4 cultures, and large lancet-shaped diplococci in 2 cultures. A series of subcultures were made from broth to broth and from broth to blood-agar plates. Broth from cultures containing fine streptococci revealed fine streptococci only, and broth from the culture having the longer coccus showed a similar large coccus. However, the blood agar plates from all 6 broth cultures contained hemolytic colonies only. While this suggested mutation, there was a possibility that a green as well as a hemolytic colony might have been picked and transferred to the stock culture, since the colonies were all minute; or there was the possibility that the colony might have contained the 2 types of coccus, though we did not get the impression from films that single colonies contained the 2 types. To rule out such possibilities it was decided to work with colonies from single cocci. By means of the Barber [15] technic a

[12] Ibid., 1921, 93, p. 96; 94, p. 107; 1923, 99, p. 366.
[13] Centralbl. f. Bakteriol., I, O., 1922, 88, p. 352.
[14] Ibid., 1912, 61, p. 86.
[15] J. Infect. Dis., 1908, 5, p. 380.

single coccus of the streptococcus viridans type was picked and planted on a blood-agar plate. One green colony resulted. This colony was transferred to broth, and its morphology and its cultural characteristics were similar to the 3 green-producing streptococci previously described. A single coccus of the streptococcus hemolyticus type was finally procured and transplanted to a blood-agar plate. The cultural characteristics of this coccus were similar to those of the stock cultures of streptococcus hemolyticus A, B, and C strains.

The pure line streptococci have been isolated for one and one-half year, and their cultural characteristics have been constantly observed. Cultural methods other than the routine subcultures have been used to induce the splitting off of variants. For example, young cultures have been plated on blood-agar; old cultures, both from the ice-box and the incubator, have been plated on blood-agar; series of varying numbers of generations of a strain have been transferred from broth to broth and then plated on blood-agar; generations have been transplanted from blood-agar plates to blood-agar plates; generations have been alternated from broth to blood, to broth, etc. Heated blood-agar plates, hemolyzed blood-agar, and dextrose agar have not been used in this attempt to change hemolytic to green colonies, since our experience has been that of Clawson,[8] Rosenow[7] and many others that this can invariably be accomplished and that it is not a true mutation, in that the hemolytic quality reappears when a colony from such medium is transplanted to the standard fresh blood-agar plates.

In addition to the streptococcus viridans, the third positive blood culture contained both hemolytic and anhemolytic colonies from the beginning. It was impossible to maintain the hemolytic and the anhemolytic strains separately, since a broth culture made from one type transferred to blood-agar might contain a mixture of the hemolytic and anhemolytic colonies, or the hemolytic colonies alone, or the anhemolytic colonies alone. The anhemolytic type of colony progressively increased, until about 7 months after the isolation of the 4 strains of hemolytic streptococcus cultures all appeared anhemolytic on blood-agar plates. The transition, with the exception of the hemolytic streptococcus from the third positive bloodculture was abrupt. The sugar reactions remained constantly those of the hemolytic streptococcus after the hemolytic quality was lost. These streptococci have remained anhemolytic in spite of attempts to change them into the hemolytic type for a period of almost 2 years. Then 3 hemolytic colonies appeared on a plate inoculated with anhemolytic streptococcus, pure line strain.

These colonies were transferred to broth and replated on blood agar, and anhemolytic colonies only resulted. The sugar reactions from a broth culture made from the hemolytic colonies were identical with the usual reactions of the hemolytic and the anhemolytic organisms. The succeeding generations have all been anhemolytic. The green-producing streptococci have remained constant since they were first isolated.

Discussion of Bacteriology.—Morphologically the 2 strains are different From the sugar reactions there are 2 strains. From the physical reaction on blood-agar there are 3 distinct types, green hemolytic and anhemolytic. Considering that we started with an anhemolytic strain, it is possible that the 2 fractions, a hemolytic and a green, were split off from the anhemolytic type, that after the cleavage the 2 varieties remained constant, and that the hemolytic quality disappeared as virulence was lost. This possibility is augmented by the fact that the third culture, obtained at a time when the patient's agglutinin titer for the hemolytic streptococcus was high, contained anhemolytic colonies. It has been thought by many investigators that hemolytic streptococci were prone to become anhemolytic or green as their virulence decreased. The sugar reactions were constant over a period of 2 years for the green streptococcus and for the hemolytic streptococcus and its anhemolytic descendant. The other possibility is that the original blood-agar streaked plate grew so slowly that the hemolytic or green-producing quality did not appear until after the first 24 hours; and as we have mentioned before, the streaks did not appear green or slightly hemolytic until after 48 to 72 hours. If the latter is correct, it would seem probable that we had 2 distinct strains of streptococci, a viridans strain and a hemolytic strain that lost its hemolyzing quality and thereafter remained almost constantly anhemolytic.

Brief Animal Passage.—The effect of brief animal passage was tried on the 2 strains, Streptococcus hemolyticus and Streptococcus viridans, as soon as they were isolated from the original positive blood culture. The technic used was essentially that described by Schnitzer and Münter [12] and previously used by one of us.[16]

A sublethal dose of an 18-hour broth culture of each streptococcus was injected intraperitoneally under aseptic conditions into each of 5 mice. Four mice were killed, 1 each hour, and the fifth mouse was killed the next day. Stained films of the peritoneal fluid of each mouse were examined, and ascites dextrose meat-infusion broth cultures and blood-agar plate cultures were made from the peri-

[16] Howell, Katharine M.: J. Infect. Dis., 1924, 34, p. 117.

toneal fluid, from the heart's blood, and from the ground kidney tissue. The films from the peritoneal fluid of the mouse injected with the hemolytic streptococcus contained chains of small round cocci varying from 4 to 12 or more cocci per chain. There was considerable variation in the morphology of these cocci, many appearing swollen. The staining quality was also irregular, in that gram-negative and gram-positive cocci appeared in the same chains. Smears from the mice killed at the end of 1 and 2 hours contained a few leukocytes and many extracellular cocci, and showed little phagocytosis. In films taken after 2 hours there were more leukocytes and many phagocytized cocci. The blood-agar plates contained hemolytic colonies only, and the blood-agar plates inoculated from the 24-hour broth culture contained hemolytic colonies only. The culture of the streptococcus viridans was similarly injected into 5 mice, and the mice killed and cultured. Diplococci and a few short chains of cocci were seen in the films. There were few leukocytes, and practically no phagocytosis was observed. The cultures of the organs and peritoneal fluid produced green colonies with the exception of 1 colony with a narrow zone of hemolysis that appeared on the plate inoculated with kidney tissue. Subcultures of this colony did not grow.

When pure line cultures of Streptococcus anhemolyticus and Streptococcus viridans were obtained from single cocci, a sublethal dose of the second generation of each (broth culture) was injected intraperitoneally under aseptic conditions into each of 6 mice. As in the previous tests, the mice were killed, 1 each hour, the usual films and cultures were made from the organs, and the cultures were examined after 24 hours. The fine, long chained streptococcus, which at this time was anhemolytic, did not split off either hemolytic or green variants; nor did the green coccus produce hemolytic or anhemolytic variants. These experiments were repeated with the third generation after isolation from single cocci. No variants occurred in this series.

In the next series of experiments, undertaken several months later, the dose was modified. The lethal dose of all the streptococcus stock cultures at this time was about 1 cc. Five mice were used in each test. Into one set of mice 0.5 cc. of Streptococcus viridans, A, pure line, was injected; into another set of mice 0.25 cc. of Streptococcus viridans from the second positive blood culture, B and into a third set 0.125 cc. of Streptococcus viridans from the third positive blood culture, C. Green colonies only appeared on all the plates but 3. The plates implanted with heart's blood of the mouse killed at the end of 1 hour and of the mouse killed at the end of 4 hours from the streptococcus viridans A series, had a few hemolytic colonies. There were also a few hemolytic colonies on the plate made from kidney tissue in the streptococcus veridans C group. Films from these hemolytic colonies had the lancet-shaped diplococcus typical of the A, B. and C streptococcus viridans stock cultures. Hemolytic colonies were transplanted to broth, and after 18 hours the broth cultures were plated on blood agar. The resulting colonies were all green. The sugar reactions of the broth cultures into which the hemolytic colonies were transferred were typical of the large type of organism. Then similar tests were made with anhemolytic streptococcus strains. The kidney cultures from the mice killed at the end of 2 and 3 hours from the group of mice receiving 0.125 cc. contained hemolytic streptococci. The peritoneal and the heart cultures in the group receiving 0.25 cc. contained the hemolytic and anhemolytic colonies in the mice killed after 1 and 2 hours. In the group receiving 0.5 cc., the heart culture from the mouse killed at the end of 1 hour and the kidney culture from the mice killed at the end of 2 hours contained hemolytic streptococci. The blood-agar plates from the rest of the series had the anhemolytic type of colony only. The hemolytic quality of the streptococci was transient.

since after the second generation no more hemolytic colonies were obtained. Broth cultures (second generation) inoculated in diagnostic sugars gave the typical fermentation reactions of the fine streptococcus.

Several months after the last experiments described, we attempted to raise the virulence of the anhemolytic and the viridans pure lines by animal passage. Many white mice were used, and the types of colonies were at the same time carefully observed on blood-agar plates, but in no instance were constant variants obtained.

Summary of the Effects of Brief Animal Passage.—Freshly isolated organisms and early generations from single cocci apparently did not split off fractions. The dosage apparently was not a determinant factor since one-half, one-quarter, and one-eighth of the sublethal dosage all produced a few transitory variants. Smaller doses did not produce variants. At no time were variants noted in mice killed later than at the end of the third hour. Cleavage did not depend upon the organ cultured, since cultures of the heart's blood, peritoneal fluid, and finely ground kidney tissue all produced transitory variants. The viridans strains were almost constant. The hemolytic strain after the hemolytic quality was first lost, produced only a few hemolytic fractions that quickly reverted to the anhemolytic type.

IMMUNOLOGIC REACTIONS

The immunologic relations of the bacteriologically different strains of streptococci were investigated. At the time that the donors were immunized by injections of streptococcus vaccine it was found that there was good immunity response, as measured by agglutinin, opsonin, and complement-fixation antibodies to the streptococcus antigen. Agglutinin for the hemolytic streptococcus appeared sooner in the serums of the 5 donors than it did for the streptococcus viridans, but after immunization was established the titer was practically the same for each streptococcus antigen. When there was a drop in agglutinin titer, in either the recipient's or the donor's serums, it always occurred first for the hemolytic streptococcus. Opsonin and complement-fixation antibodies also tended to be more readily elicited by the hemolytic streptococcus.

Rabbits were immunized with each streptococcus strain in order to observe the results of cross immunity tests. The streptococcus suspensions used for vaccination, agglutination, absorption, opsonic and phagocytic indices, and complement-fixation were prepared as follows: 18-hour growths from ascites meat-infusion, dextrose broth, P_H 7.6, were centrifuged, the supernatant removed, the precipitated streptococci washed in salt solution, and resuspended in salt solution so that there were approximately 1 billion streptococci per cc. The suspensions

were heated at 56 C. for 30 minutes. Neither streptococcus clumped spontaneously in broth mediums, so that smooth bacterial suspensions were easily obtained.

The inactivated serums of 2 half-grown normal rabbits were tested for agglutinins. The macroscopic agglutination method was used. To varying dilutions of serum placed in small test tubes, an equal amount of standard bacterial suspension was added. The tubes containing the mixtures were incubated for 2 hours in a hotwater bath at 56 C. The results were read, and the highest dilution of serum that agglutinated the bacteria was noted. The tests were then left in the ice-box over night, and a final reading was taken the next morning. Neither rabbit's serum agglutinated the suspension of the viridans streptococcus, but both serums agglutinated the suspension of hemolytic streptococcus in dilution of 1 : 80. The agglutination tests were repeated with all the hemolytic strains, and the same results were obtained. Eight normal rabbits were tested before 2 were found whose serums did not agglutinate suspensions of the hemolytic streptococcus. One of these rabbits was vaccinated by intravenous injection of the hemolytic streptococcus, B strain, and the other rabbit by a similar injection of the streptococcus viridans, B strain. The serums of both rabbits had a higher agglutinin titer for the suspension of hemolytic streptococcus than for the suspension of the viridans organism. Both serums agglutinated the viridans suspension, but not so quickly nor in such high dilutions as they did the hemolytic streptococcus suspension. The viridans suspension was agglutinated by a higher dilution of the homologous serum than by the heterologous serum. These tests showed such lack of specificity that no further work was done with these 2 rabbits.

Two fresh rabbits were injected with the streptococcus vaccine. The agglutinin reactions were as nonspecific as those of the first group. A third set of rabbits were immunized as before. In these tests the hemolytic streptococcus immune serums tended toward specificity, and the streptococcus viridans immune serum toward nonspecificity. The hemolytic streptococcus B immune serum agglutinated suspensions of the hemolytic strains A and C in as high dilution as it did its homologous suspension B. Streptoccocus viridans B immune serum agglutinated suspensions of viridans A, B, and C in about the same dilution.

At this period, about 7 months after isolation, the long, fine chained streptococcus that had been hemolytic on blood-agar plates lost the hemolytic quality. Morphologically the anhemolytic coccus was identical to the hemolytic coccus, and the fermentation reactions of the hemolytic and anhemolytic cocci were identical. Serum, human and rabbit, containing agglutinin, opsonin, and complement-fixation antibodies for the hemolytic streptococcus antigen, gave a similar reaction with the anhemolytic streptococcus antigen. In order to compare the antibody response of this anhemolytic streptococcus with that of its hemolytic predecessor, 1 rabbit was immunized with this anhemolytic strain; another rabbit with equal parts of the anhemolytic strain and the viridans strain; and a third rabbit was immunized with Streptococcus viridans alone. As in the case of the hemolytic streptococcus, a high antibody response was quickly elicited by this anhemolytic antigen. The agglutinin titer of the anhemolytic immune serum with both the anhemolytic and the hemolytic suspensions was 1 : 20,480. The titer of the viridans strain was 1 : 320 with the anhemolytic streptococcus antigen. The antiserum of the rabbit injected with a mixed antigen had an agglutinin titer of 1 : 10,240 for the hemolytic and anhemolytic antigens and a titer of 1 : 640 for the viridans antigen. The rabbit immunized with Streptococcus viridans alone had a titer of 1 : 1280 for the viridans and 1 : 320 for the hemolytic and anhemolytic antigens. Immunologically as well as bacteriologically, therefore, the anhemolytic streptococcus appeared identical to the hemolytic

streptococcus. The hemolytic strain and its anhemolytic descendant could not be differentiated by virulence tests since the hemolytic strain had been almost avirulent for white mice for several months before the hemolytic quality was lost.

Six more rabbits were immunized before the attempt was given up to stimulate a specific immune serum or an immune serum with so high a titer for both streptococcus antigens that it would make a satisfactory immune serum for absorption tests. After the immunity curve of a rabbit immunized with anhemolytic streptococcus vaccine had dropped, it was reinjected with a vaccine of the streptococcus viridans strain. The resulting agglutinin titer for the streptococcus viridans was only 1 : 10, while there was a restimulation of the anhemolytic streptococcus agglutinin titer to 1 : 5120.

The rabbits did not lose their immunity for these streptococcus strains gradually but abruptly. Rabbits that had been under observations for several months and that had little variation in agglutinin titer in that time might have an abrupt drop from 1 : 5120 or even from 1 : 20,480 to 0 within the course of a few days. After this drop in immunity had taken place, it was frequently impossible to restimulate the streptococcus antibodies although various methods were used, such as injecting attenuated cultures or living virulent organisms, varying the doses, etc. It had been noticed that, when the donors who had been immunized with these streptococci had had a decrease in antibody titer, antibodies could be elicited in fresh donors sooner than they could be restimulated in the previously immune donors. We have no explanation for this abrupt decrease in antibody nor for the difficulty in restimulating the antibody.

Erythrocyte, leukocyte and differential blood counts and hemoglobin determinations were made on a number of rabbits at the same time that the agglutinin titer was measured. No correlation was apparent between the blood count and the agglutinin titer. In fact, the blood counts were unusually stable for rabbits.

The antistreptococcus serums of some of the immunized rabbits were tested for opsonin and complement-fixation antibodies in addition to agglutinins. Opsonin was estimated by the opsonic index method. The opsonic curves paralleled those of the agglutinins. The opsonin in the immune serum was even less specific than the agglutinin.

Complement-fixation tests were made according to the classical Wassermann test (one-tenth method). Hot water incubation was used 1 hour for the first incubation and 30 minutes for the second. The readings were made and recorded immediately after the second incubation. A weakly positive reaction, + or + +, indicated that there was complete inhibition of hemolysis with ¼ or ⅛ of the anticomplementary unit of streptococcus antigen. A strongly positive reaction, + + + or + + + +, indicated that there was complete inhibition of hemolysis with 1/64 or less of the anticomplementary unit of a streptococcus antigen. The complement fixation tests showed considerable cross fixation. For example, if an immune serum was strongly positive with anhemolytic streptococcus antigen, it would probably be + + or + + + with the streptococcus viridans antigen. Many of the complement fixation tests could not be read because of the tendency of the rabbit serum controls to be anticomplementary.

Agglutinin absorption tests were made in the following way: The immune serum was agglutinated with each strain of streptococcus and the titer noted. One portion of the immune serum was then absorbed with the viridans strain and another portion with the hemolytic strain or with its anhemolytic descendent. Each absorbed serum was then tested for agglutinins with the viridans, hemolytic, or anhemolytic suspension. The absorption tests on the rabbit serum were not satisfactory because a serum of high titer for each strain could not be obtained.

After a single coccus of the streptococcus viridans A type and a single coccus of the anhemolytic streptococcus A had been picked and cultured, a vaccine was made from an early generation of each strain. Since the vaccinated rabbits had given such irregular immunity reactions, 3 healthy young persons were chosen for immunization. One was vaccinated with an anhemolytic streptococcus pure-line strain, a second with a streptococcus viridans pure-line strain, and a third with equal parts of the two vaccines. None had a systemic reaction; all had about the same degree of local reaction, an area of redness and induration about 3 cm. in diameter.

The normal serum of the volunteer for the streptococcus viridans vaccine had no agglutinin, opsonin, or complement-fixation antibody present in his serum for either streptococcus strain before he was immunized. Fourteen of 50 leukocytes were phagocytic for the viridans, and 7 leukocytes were phagocytic for the anhemolytic streptococcus. Three doses of vaccine were given at 6 to 8 day intervals. Six days after the first injection of vaccine, the agglutinin titer for each strain was 1:20. The opsonic index for the viridans was 1.7 and for the

TABLE 1

The Antibody Response of a Normal Person to Streptococcus Viridans Vaccine

Date	Cc. of Streptococcus Viridans Vaccine 1 Billion per Cc.	Agglutinin		Opsonic Index		Phagocytic Index		Complement Fixation	
		Viridans	Anhemolyticus	Viridans	Anhemolyticus	Viridans	Anhemolyticus	Viridans	Anhemolyticus
10/ 3/24	0.5	0	0	0	1	14	7	0	0
10/ 9/24	1	20	20	1.70	6	6	7	0	0
10/15/24	1	160	160	7.00	2	6	10	0	0
10/23/24	...	160	5,120	3.50	5.5	5	5	0	0
10/28/24	...	1,280	640	6.00	2.3	4	8	0	++
11/ 3/24	...	5,120	5,120	6.50	4	10	12	0	+
11/ 6/24	...	640	160	6.00	5	7	11	0	+
2/16/25	1	10	40	3.25	3.72	4	8	+	++
2/24/25	...	80	20	1.03	1.09	0	2	0	0
2/26/25	...	20	0	1.00	1.8	1	2	+	0
2/27/25	1	.....	.....	....	....	..	..	..	..
3/ 4/25	...	40	0	7.00	2	5	10	0	0
3/ 4/25	...	160	40	7.00	5.6	9	1	0	0

anhemolyticus 6. The phagocytic index for the viridans was 6 and for the anhemolytic streptococcus 7. The complement-fixation tests were negative. Twenty days after the first dose of vaccine, the agglutinin and opsonin were higher for the heterologous strain than for the homologous strain, the phagocytic index was 5 for each strain, and the complement-fixation tests were still negative. After 24 days the complement-fixation test was weakly positive with the heterologous, but still negative with the homologous antigen. Two absorption tests were made with the immune serum from this volunteer. The first serum tested agglutinated the viridans at 1:640 and the anhemolytic streptococcus at 1:128; the second serum had an agglutinin titer of 1:1280 for the viridans and 1:640 for the anhemolyticus. In each instance when the serum was absorbed with the anhemolytic streptococcus, the agglutinins were also removed for the streptococcus viridans; and when the serum was absorbed with the streptococcus viridans, the agglutinins were removed for the anhemolytic streptococcus as well.

Three and one-half months after the height of the immune curve the volunteer was bled again, and his serum was tested for residual immune bodies. The agglutinin for the homologous strain was at this time 1:10, the opsonic index 2,

the phagocytic index 4, and the complement-fixation test 1 +. For the heterologous strain the agglutinin titer was 1 : 40, the opsonic index 0, the phagocytic index 8, and the complement-fixation reaction 1 +. It is apparent, therefore, that immune bodies not only appeared first for the anhemolytic streptococcus (heterologous strain) but also persisted for a longer period. This volunteer received 2 more doses of vaccine. As in the case of the donors and of the immunized rabbits it was difficult to restimulate agglutinin, 1:160 being the highest titer reached. The opsonic and the phagocytic indices were increased, but the complement-fixation antibodies again became negative (table 1).

The volunteer immunized with the anhemolytic streptococcus vaccine had no antibody for either streptococcus strain before injection. Six days after the first dose of vaccine (½ billion) the agglutinin titer for the anhemolytic streptococcus was 1 : 10,240, the opsonic index 6, and the phagocytic index 8. The agglutinin titer for the streptococcus viridans was 1 : 10, the opsonic index 3.6, and the phagocytic index 4. No complement-fixation antibodies could be detected for either streptococcus antigen. The immune bodies reached their height 1

TABLE 2

THE ANTIBODY RESPONSE OF A NORMAL PERSON TO STREPTOCOCCUS ANHEMOLYTICUS VACCINE

Date	Cc. of Streptococcus Anhemolyticus Vaccine 1 Billion per Cc.	Agglutinin		Opsonic Index		Phagocytic Index		Complement Fixation	
		Viridans	Anhemolyticus	Viridans	Anhemolyticus	Viridans	Anhemolyticus	Viridans	Anhemolyticus
10/14/24	0.5	0	0	1.00	1	0	1	0	0
10/23/24	1	10	10,240	3.60	6	4	8	0	0
10/28/24	1	10	10,240	1 00	1	2	2	0	0
11/ 7/24	...	10	640	2.00	4	3	9	0	0
11/10/24	1.5	..	.....	....	....	..	..	..	..
11/18/24	...	20	20,480	3.00	6.5	3	13	0	..
2/16/25	1	40	160	5.30	4.36	6	11	0	+
2/24/25	...	0	10	1.16	3.33	4	6	0	0
2/26/25	...	0	0	0.90	2.1	2	2	0	0
2/27/25	1	..	.....	....	....	..	..	..	..
3/ 4/25	...	20	160	5.00	2.5	4	10	0	0
3/ 9/25	...	20	2,560	7.00	6	3	14	0	0

month after the first dose of vaccine. Three months after the height of the immunity curve the volunteer was bled, and his serum tested for streptococcus antibodies. As in the case of the volunteer who had been immunized with the streptococcus viridans, the agglutinin titer had dropped, the opsonic and phagocytic indices had remained high, and the complement-fixation reaction was higher than it had been when observation had been discontinued. This volunteer received 2 more doses of anhemolytic streptococcus antigen. The antibodies were restimulated with difficulty, agglutinin not increasing in titer for one month (table 2).

Absorption tests were made with serums from this immunized volunteer. The results were as follows: In the first test the agglutinin titer was 1 : 20,480 for the anhemolytic streptococcus and 1 : 40 for the streptococcus viridans; absorption with the anhemolyticus left a serum that agglutinated the viridans 1 : 20. When the serum was absorbed with the viridans, the agglutinin for the anhemolyticus was also removed. A second test was similar to the first, with the exception that all the agglutinin for the viridans was absorbed from the anhemolytic streptococcus serum.

The volunteer who received equal parts of the 2 streptococcus vaccines had a weakly positive complement-fixation reaction before immunization, but no other antibodies could be detected. Agglutinin appeared first and rose higher for the anhemolytic streptococcus strain; the phagocytic index followed a similar course, but the opsonic index was higher for the streptococcus viridans. The complement-fixation test remained weakly positive with a streptococcus viridans antigen, but it became strongly positive with the streptococcus anhemolyticus antigen. An absorption test was made when the agglutinin titer was 1:20,240 for the anhemolytic streptococcus and 1:2560 for the viridans. Absorbing the immune serum with either streptococcus organism removed the agglutinin for both strains. This volunteer was not available after 3 months, so this serum could not be tested for residual immune bodies (table 3).

The results of these immune reactions were checked by the following method. After the first tests were made, as soon after the person was bled as possible, the remainder of the serum was stored in the ice-box; after the last bleeding, the entire set of serums was reheated and the

TABLE 3

The Antibody Response of a Normal Person to Streptococcus Viridans and Streptococcus Anhemolyticus Vaccine

Date	Cc. of Streptococcus Viridans and Anhemolyticus Vaccine	Agglutinin		Opsonic Index		Phagocytic Index		Complement Fixation	
		Viridans	Anhemolyticus	Viridans	Anhemolyticus	Viridans	Anhemolyticus	Viridans	Anhemolyticus
10/20/24	1	0	0	0.75	0.5	.56	.26	+	+
10/24/24	1	20	320	1.20	6.0	.60	.70	+	+++
11/ 3/24	1.5	640	1,040	13.50	5.0	.20	.23	+	++
11/13/24	...	2,560	20,240	.....	...	...	...	++	+++

immune tests were repeated on all the serums at the same time. There was remarkably little difference in the 2 readings; the results tabulated, however, are those of the first reading.

Protection Tests: Toxins were prepared from each streptococcus strain. The volunteers were given an intracutaneous dose of each toxin and its control (heated toxin). The controls were negative, and the 2 toxins gave identical results with all the volunteers, i. e., an area of redness 4 to 6 cm. in diameter.

The protective action of the immune serum of the 3 volunteers was tested in white mice. To do this it was necessary to raise the virulence of the 2 streptococcus strains, since they had both become almost avirulent for white mice. By successive animal passage the virulence of Streptococcus viridans was increased, so that 0.5 cc. of an 18-hour broth culture uniformly killed a mouse in less than 24 hours.

Six mice then received injections as follows:

Mouse 1 received 0.5 cc. streptococcus viridans.

Mouse 2 received 0.5 cc. streptococcus viridans and 0.5 cc. of the streptococcus viridans antiserum.

Mouse 3 received 0.5 cc. streptococcus viridans + 0.5 cc. of streptococcus anhemolyticus antiserum.

Mouse 4 received 0.5 cc. streptococcus viridans + 0.5 cc. of normal serum.

Mouse 5 received 1 cc. of streptococcus viridans antiserum.

Mouse 6 received 1 cc. of streptococcus anhemolyticus antiserum.

Mice 1 and 3 died within 24 hours after injection. Mice 2, 5, and 6 were living and apparently well 24 hours after injection. Mouse 4 appeared ill at the end of 24 hours but recovered after several days.

After successive animal passage 1 cc. of an 18-hour broth culture of the Streptococcus anhemolyticus strain killed a white mouse in 24 hours, and 0.5 cc. of the suspension killed mice part of the time.

Six mice were then injected as follows:

Mouse 1 received 1 cc. of anhemolytic streptococcus.

Mouse 2 received 1 cc. of anhemolytic streptococcus, + 1 cc. of anhemolytic streptococcus antiserum.

Mouse 3 received 1 cc. of anhemolytic streptococcus, + 1 cc. of the streptococcus viridans antiserum.

Mouse 4 received 1 cc. of anhemolytic streptococcus, + 1 cc. of normal serum.

Mouse 5 received 1 cc. of anhemolytic streptococcus antiserum.

Mouse 6 received 1 cc. of the streptococcus viridans antiserum.

In 24 hours mouse 1 was dead, mice 2, 5 and 6 were living and well, and mice 3 and 4 were ill. These mice were apparently well after several days. Streptococci used in the protection experiments were washed organisms. These protective tests were not considered conclusive because of the low-grade virulence of the organisms and because of the fewness of the tests carried out. However, they suggest that the homologous antiserum protected the mice in a higher degree than did the heterologous antiserum or normal serum.

Virulence tests on rabbits according to the method described by Rivers [17] were tried. Rabbits received injections intracutaneously under aseptic conditions as follows: in the midline normal serum plus anhemolytic streptococcus, on the left side anhemolytic streptococcus immune serum plus anhemolytic streptococcus, and on the right side streptococcus viridans antiserum plus anhemolytic streptococcus. Three dilutions of serum were used in each test. After 24 hours all 9 points of injection were elevated and red, and they showed no difference in appearance. After 5 or 6 days all 9 regions became pustular. Another rabbit was similarly injected with the streptococcus viridans instead of the anhemolyticus. The 9 resulting lesions were identical. The virulence tests were modified by mixing the streptococcus strain and the serum and leaving

[17] J. Exper. Med., 1925, 41, p. 179.

the mixture at room temperature 1 hour before injection. Twenty-four hours after intracutaneous injection of these mixtures the 9 resulting lesions on each rabbit were identical, and after 5 or 6 days all the lesions became pustular.

Discussion of Immunologic Reactions.—The immunity tests showed such variability and nonspecificity that they did not materially aid in deciding whether we were dealing with 2 distinct strains of streptococcus or a single mutating strain. Summarizing the immunity tests we had the following, rather contradictory results:

Agglutinin: The streptococcus agglutinin in the serum of the immunized persons or rabbits, whether vaccinated with the single or combined streptococcus strains appeared first and rose higher for the hemolytic streptococcus or its anhemolytic variant than it did for the streptococcus viridans. Streptococcus viridans suspension was agglutinated and in a higher dilution by the streptococcus viridans antiserum than it was by hemolytic streptococcus antiserum. In most of the serums the hemolytic streptococcus antibody could be detected several months after the person had received the last vaccine. Streptococcus viridans immune bodies had usually disappeared in this serum. If streptococci can be classified by agglutinin reactions, as Dochez, Avery and Lancefield[18] and others believe, the results obtained with these streptococci would suggest that they belong to one strain, Streptococcus hemolyticus. The agglutinin reactions of the hemolytic streptococcus and its anhemolytic variant were identical. This finding agrees with a report made by Valentine and Krumwiede.[19] A hemolytic streptococcus which had been constantly hemolytic on artificial media for over a year suddenly split off an anhemolytic variant. After cleavage this variant remained constant for over 50 transfers. The 2 forms of this streptococcus remained morphologically, culturally, and immunologically identical.

Agglutinin Absorption: The absorption test indicated the presence of only a single agglutinin. Whenever either immune serum was absorbed with one streptococcus type the agglutinin for the other type was either completely or almost completely removed.

Opsonins: The opsonic indices of the immune streptococcus serums were high, a finding contrary to that of Weaver,[19] who considered that the unsatisfactory result of treatment in streptococcus infections by antiserum was due to the low opsonic content of the serum. The opsonic indices in our tests did not suggest specific differences. According to the observations of Tunnicliff[20] on the specificity of the opsonic test in streptococcus antiserum, this finding would point toward one rather than two distinct strains.

Phagocytic Index: The phagocytic activity of the leukocytes for the 2 types of streptococci was high, but it was not specific.

Complement-Fixation Antibodies: Complement-fixation antibodies for the 2 types of streptococci were elicited with difficulty. They appeared late in the course of immunization, and they rarely gave strongly positive reactions, either with the homologous or the heterologous antigen. This was a disappointment, since the reports of Kinsella and Swift,[21] Kinsella,[22] Floyd and Wolbach,[23] and

[18] Ibid., 1919, 30, p. 179.
[19] J. Am. M. A., 1921, 76, p. 34.
[20] Ibid., 1920, 75, p. 1339.
[21] Ibid., 1918, 28, p. 169.
[22] Ibid., 1918, 28, p. 181.
[23] J. M. Research, 1914 29, p. 493.

others suggest that the complement-fixation reaction may prove a possible method of grouping streptococci, though in our own previous experience[24] we had not found this to be the case.

Toxin: The toxins for the two strains were nonspecific.

Protection: The protection experiments appeared to indicate specific differences between the 2 strains. The homologous immune serum protected mice against the intraperitoneal injection of its own strain, while neither the heterologous immune serum nor the normal serum conferred any protection. Cowan,[25] in her protective experiments on mice and rabbits, found that homologous immune streptococcus serum offered more protection than a heterologous immune serum

It is difficult to arrive at a definite conclusion as to whether there were two distinct strains of streptococci or a single one undergoing mutation. In the latter case it is necessary to suppose that the original single strain, which was anhemolytic, split off hemolytic and green-producing variants and that the hemolytic variant later reverted to the anhemolytic type. Objections to this possibility are that the morphology and sugar reactions were constantly different from the first and that the protective experiments also indicated 2 strains. The morphology, the bacteriology, and the animal passage experiments make it appear more probable that there were 2 distinct strains from the beginning and that the hemolytic quality only was lost by the hemolytic strain. The main objection to this view is that the agglutinin absorption tests plainly indicated one strain. It may be that immunity reactions in the streptococcus group are not specific enough to classify streptococci, or that the parasitic existence of 2 originally different streptococci in the same lesion had so modified their immunity reactions that the 2 strains could not be immunologically separated.

In a previously reported study[26] of a hemolytic and a viridans streptococcus isolated from the same aortic valve lesion, it was noted that only the green variety was obtained from a second, more acute lesion situated near the mouth of the pulmonary artery. In another case of endocarditis which has recently come to necropsy, the clinical course of the disease was that of subacute bacterial endocarditis, with which Streptococcus viridans is so frequently associated. Repeated blood cultures were negative. The clinical picture finally changed to that of an acute, malignant endocarditis; during this terminal stage Streptococcus hemolyticus was isolated from the blood. At necropsy the mitral valve was the seat of a large, fresh lesion of the acute, vegetative type. Two cases in each of which 2 distinct strains of streptococcus were isolated

[24] Howell, Katharine M.: J. Infect. Dis., 1918, 22, p. 230.
[25] Brit. J. Exper. Path., 1924-25, 5, p. 226.
[26] Howell, Katharine M.: J. Infect. Dis., 1922, 30, p. 299.

and a third case which began clinically as a subacute bacterial endocarditis but ended as an acute malignant endocarditis with isolation of hemolytic streptococcus from the blood, suggest that infectious endocardial lesions may be initiated by one species of organism, with which a second strain or species may later become associated, transforming an originally more slowly progressing condition into an acute and more rapidly fatal one.

SUMMARY

From the blood of a case which was clinically one of acute malignant endocarditis there were isolated on 3 successive occasions 2 bacteriologically distinct organisms, Streptococcus hemolyticus and Streptococcus viridans. The 2 strains were morphologically different; the morphologic differences have remained constant over a period of 2 years. The sugar reactions, which were also different, have remained constant for the same period.

The hemolytic streptococcus strain 7 months after isolation lost its hemolyzing quality and remained constantly thereafter anhemolytic. One constant mutation, therefore, occurred.

Brief animal passage indicated that the 2 strains were different. It was impossible by this experimental procedure to split off a viridans variant from the hemolytic streptococcus or its anhemolytic form, or the reverse. Transient variants occasionally occurred.

The immunologic reactions, although variable, suggested that there was only a single strain, the hemolytic streptococcus. It is possible that the parasitic growth of 2 closely related bacterial strains may so alter their immunologic reactions that differentiation by such reactions becomes impossible.

Protection experiments with immune serums indicated that the two strains were different.

DIFFERENCES IN PEROXIDE PRODUCTION AND METHEMOGLOBIN FORMATION OF GREEN (ALPHA) STREPTOCOCCI *

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Blood plates streaked † on the surface with throat material frequently may show green colonies of the size characteristic for the members of the streptococcus-pneumococcus group which vary considerably in general appearance. Some colonies, usually of moderate size, may be surrounded by a wide, bright green zone, while others may be smaller and the discolored area narrow and pale green. When studied in blood agar pour plates according to the method and criteria of Brown [1] all of these colonies fall within the alpha group. That these variations are not necessarily related to the species differences between green streptococcus and pneumococcus was determined by isolation and identification of the colonies. It seemed of interest, therefore, to ascertain whether any difference in the behavior of the strains upon red blood cells could be demonstrated by other methods. Furthermore, if a positive result could be correlated with a fundamental difference in the chemical (oxidation-reduction) or biological action of the two types observed on the blood plate, it might serve as a simple means of differentiating subgroups of the green (alpha) or viridans streptococci.

The relation of green discoloration on blood agar plates to the formation of methemoglobin as evidenced by the browning which occurs when broth cultures of pneumococci and green producing streptococci are incubated with washed red blood cells in the test tube has been recognized for many years. This phenomenon has been studied by many workers, namely Butterfield and Peabody,[2] Cole,[3] Lyall,[4] Blake,[5]

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† Although the streaked blood agar plate is superior to the dilution pour plate in making the preliminary differentiation between X and Y green streptococci, it is inferior to the latter in general diagnostic procedures involving streptococci.

[1] Monograph No. 9, Rockefeller Inst. for Med. Research, 1919.

[2] J. Exper. Med., 1913, 17, p. 587.

[3] Ibid., 1914, 20, p. 363.

[4] J. M. Research, 1914, 30, p. 487.

[5] J. Exper. Med., 1916, 24, p. 315.

McLeod and Gordon,[6] Neill and Avery[7] and others. In a recent publication, Neil[8] gives a historical review of the extensive literature on this subject. The recognition of this relationship between green pigmentation and methemoglobin formation led to a series of test tube experiments, which are detailed in this paper. For convenience in recording the findings, the two varieties are designated X and Y.

APPEARANCE OF VARIETIES ON BLOOD MEDIUMS

(a) Surface colonies on streaked blood plates (1 cc. defibrinated sheep blood, 12 cc. veal infusion agar). Individual colonies of the X variety after 24-48 hours incubation are surrounded by an area of bright green discoloration which seems to diffuse into the blood. As a rule, they resemble pneumococcus colonies in size. Y colonies are generally smaller and the growth which is often definitely raised is surrounded by a narrow, pale green zone which at times may be difficult to detect. When pure cultures are streaked heavily on the blood plate, the differences are more marked (dividing the plate into 4, 6 or 8 sections was found to be very satisfactory). After 24 hours incubation the growth of the X cultures changes the red blood cells to a bright green which may turn yellowish green after 48 hours; the Y cultures on the other hand, produce a darker or pale green discoloration of the cells with no tendency of turning yellow. The thickness of the medium may influence the appearance to some extent.

(b) In dilution blood agar pour plates observed according to the method of Brown[1] no definite distinctions between X and Y colonies can be made. Microscopically both show fixation of the red blood cells, although the X colonies are usually larger and the zone of fixed cells is wider and more dense than noted with the majority of Y strains. On refrigeration of the plates for 48 hours, subsequent to the 48 hour incubation period, both varieties exhibit a secondary zone of clearing beyond the fixed cells. This zone around Y colonies is usually narrow and slightly hazy.

(c) On "chocolate" agar slants the action of the two varieties is quite distinct. After 24 hours incubation the surface growth of both types is slightly greenish, but by that time the chocolate color of the medium in the upper portion of the slant on which the X organisms are growing has changed to a decided yellow. In 48-72 hours this discoloration may extend to the butt of the slant. The medium on which Y cultures have grown retains its chocolate color until 48-72 hours when a faint greenish yellow tinge may be noted. The growth of both varieties on chocolate agar is luxuriant.

THE EFFECT OF BROTH CULTURES ON RED BLOOD CELL SUSPENSIONS

In studying the action of these organisms on red blood cell suspensions the original method of Lyall,[4] with a modification used by Valentine[9] in testing a hemolytic streptococcus, was followed. Graded amounts of 18 hour broth cultures (beef infusion broth, P_H 7.2-7.4 containing 1½% Witte peptone) were mixed with 1 cc. of 3% suspension

[6] J. Path. and Bact., 1922, 25, p. 139.
[7] J. Exper. Med., 1924, 39, p. 757.
[8] Ibid., 1925, 41, p. 299.
[9] J. Infect. Dis., 1922, 30, p. 610.

of washed sheep cells in 0.9% salt solution in small test tubes. When less than 0.5 cc. of culture was used, the volume was brought up to that amount by the addition of sterile beef broth. The tubes were shaken, placed in a water bath at 37 C. and observed after ¼, ½, 1 and 2 hours. Table 1 shows the result of one preliminary test.

The data presented in table 1 indicate that the action of the two organisms on red blood cells is different. The failure of the Y strain to brown the cells unless a small amount of sterile broth is added is strikingly similar to the observations made regarding the hemolytic action of an unusual strain of beta hemolytic streptococcus (Valentine [9]).

An experiment was performed with 35 cultures of green streptococci. Twenty belonged to the X variety and 15 to the Y. In this and in later experiments the method was slightly modified. Duplicate series

TABLE 1

METHEMOGLOBIN REACTIONS OF TWO STRAINS OF GREEN (ALPHA) STREPTOCOCCI

	X			Y			Control
18 hour broth culture in cc.	0.5	0.2	0.1	0.5	0.2	0.1	...
Sterile beef broth in c c.	...	0.3	0.4	...	0.3	0.4	0.5
3% sheep red blood cells in c c.	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hours at 37 C. ¼	B +	... 0	... 0	... 0	... 0	... 0	... 0
Hours at 37 C. ½	B ++	rb ±	... 0	... 0	rb Trace	... 0	... 0
Hours at 37 C. 1	B ++	B ++	B +	... 0	B +1	rb Trace	... 0
Hours at 37 C. 2	B ++	B ++	B ++	rb Trace	B ++	B ++	... 0

rb = red brown, first change in color of red blood cells.
B = brown discoloration of red cells.
++, +1, +, ± = degrees in the amount of browning.
0 = no change in bright red color of cells.

were arranged; in one tube a definite quantity of culture received a certain amount of sterile beef infusion broth, while in another tube the broth was replaced by the same amount of 0.9% or buffered salt solution (P_H 7.0). One cc. of washed, defibrinated sheep cells made up to 2% in salt solution was added to all tubes. The results obtained with six cultures are given in table 2 as representative of the actions of the X and Y strains tested. Unless otherwise noted, all tests with red blood cells were performed immediately after removing cultures from the incubator.

This experiment brought out marked differences. All of the strains designated as representative of the X variety showed evidence of browning of the red cells in both tubes after 15 minutes exposure to 37 C. in a water bath, and were completely browned at the end of one hour. The

Y strains, on the other hand, gave a definite methemoglobin reaction in the presence of sterile broth, but little or no effect on the cells in the presence of salt solution was noted. The methemoglobin formation by the Y (plus broth) occurred more slowly than in the case of the X strains; some Y cultures began to discolor the cells after ½ hour at 37 C. while others required 1 or 1½ hour. The Y strains varied as to the amount of sterile broth needed to give a strongly positive methemoglobin reaction at the end of two hours in the water bath. The majority of those studied were activated by the addition of 0.1 cc. or even 0.05 cc. of broth, although a few required as much as 0.2-0.25 cc. A beta hemolytic streptococcus used as a control completely hemolyzed the cells;

TABLE 2

REACTIONS OF SIX REPRESENTATIVE X AND Y STRAINS

	E		P		20 X		20 Y		21		65	
18 hour broth cultures	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Sterile broth	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...
Salt solution	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1
2% sheep cells, all in cc.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hours at 37 C. ¼	rb ±	rb +	rb ±	... 0	rb +	B +	... 0	... 0	rb +	B +	rb Trace	... 0
½	B +	B +1	B +	... 0	B +1	B ++	... 0	... 0	B +	B ++	rb +	... 0
1	B ++	B ++	B ++	... 0	B ++	B ++	rb ±	... 0	B ++	B ++	B +1	... 0
2	B ++	B ++	B ++	rb Trace	B ++	B ++	B +	... 0	B ++	B ++	B ++	... 0
Appearance of growth on surface blood plate	Bright green		Dark green		Bright green		Dark green		Bright green		Dark green	
Presence of peroxide * after aeration for 1 hour	++		0		++		0		+++		0	

Symbols for methemoglobin reaction as in table 1.
* Determined by the benzidine reaction, 0 indicates no reaction; ++ blue color of potato; +++ deep blue color or potato and slight blue color of surrounding fluid.

a gamma or indifferent streptococcus had no effect. None of the green streptococci showed any hemolysis in the test tube even after refrigeration of the blood tubes over night.

PRODUCTION OF HYDROGEN PEROXIDE

The production of peroxide by certain organisms and its relation to green pigment production on blood agar and methemoglobin formation has been established during the past few years (McLeod and Gordon,[6, 10] and Avery and Morgan [11]). To quote Neill and Avery [7] "The active agent in the oxidation of hemoglobin is considered to be a peroxide of

[10] Biochem. J., 1922, 16, p. 499.
[11] J. Exper. Med., 1924, 39, p. 275.

bacterial origin." These workers have shown that in order to demonstrate conclusively the presence of peroxide in bacterial broth cultures, certain prerequisites must be fulfilled; they must be exposed freely to the air in shallow layers during growth, or when grown in deep columns of broth, they must be aerated at the end of the incubation period.

The X and Y cultures frequently grew poorly in shallow layers of broth, therefore, they were cultivated in tubes containing 10 to 12 cc. At the end of the desired incubation period, they were thoroughly shaken and exposed in shallow layers at room temperature to the oxygen of the air. After 18-24 hours growth they were subjected to the benzidine test according to the method used by Avery and Morgan.[11] Invariably the X strains, the strong methemoglobin formers, gave positive peroxide reactions after an exposure for one hour, while the Y strains were completely negative even after exposure for four hours. This is in keeping with the observation of Neill and Avery[7] working with the pneumococcus, who state that "sterile extracts possessing the most marked peroxide-forming activity cause the most marked destruction of hemoglobin."

Avery and Morgan[11] tested six cultures of streptococcus viridans after 18 hours incubation and found that all gave positive peroxide tests. Hagan[12] studied the peroxide production of a single strain of streptococcus viridans and concludes that for the demonstration of peroxide "the organisms must be living and under condition which permit active exercise of their metabolic processes."

CULTURES TESTED AT DIFFERENT GROWTH PERIODS

The tests reported in the preceding paragraphs were all made with 18-24 hour cultures. Since it is reasonable to assume that the various agents concerned in the reaction may develop, persist, or disappear at different periods of the growth curve, it was deemed advisable to study, (1) the effect on the red blood cells, (2) the peroxide production, after incubation of 4, 8, 12, 18, 24, 48, and 72 hours. In order to insure uniform conditions, 10 cc. of beef broth in ordinary test tubes were inoculated with 0.1 cc. of actively growing eight hour cultures. The majority of X and Y cultures produced a moderately heavy and diffuse cloud after eight hours incubation in this broth.

Effect on Red Blood Cells.—In performing the tests, the procedure previously mentioned in the second experiment was adhered to. The data are summarized in table 3.

[12] J. Infect. Dis., 1925, 37, p. 1.

TABLE 3

METHEMOGLOBIN REACTIONS AND TESTS FOR PEROXIDE AT DIFFERENT PERIODS OF GROWTH

X Streptococcus

Hours of Growth	4		8		12		18		26		48		72	
Broth culture	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Sterile broth	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...
Salt solution	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1
2% sheep cells (all in c c.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hours at 37 C. ¼	rb Trace	... 0	rb Trace	rb ±	rb ±	B +	rb +	B +	rb ±	rb +	rb ±	rb +	... 0	... 0
Hours at 37 C. ½	B +	rb ±	B +	B +1	B +1	B ++	B +1	B ++	B +	B +1	B +	B +1	rb x	rb ±
Hours at 37 C. 1	B +1	B +	B ++	B ++	B ++	B ++	B ++	B ++	B ++	B ++	B ++	B ++	rb +	B +
Hours at 37 C. 2	B ++	B ++	B ++	B ++	B ++	B ++	... ...	... ...	... ...	... ...	... ...	... ...	B ++	B ++
Peroxide test after aeration for 5 minutes	0		0		0		±		+		2+		+	
1 hour	0		+		2+		2+		2+		3+		2+	
2 hours	+		2+		3+		3+		4+		4+		2+	

Y Streptococcus

Hours of Growth	4		8		12		18		26		48		72	
Broth culture	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Sterile broth	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...
Salt solution	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1
2% sheep cells (all in c c.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hours at 37 C. ¼	rb ±	rb Trace	rb ±	... 0	rb +	... 0	rb +	... 0	... 0	... 0	... 0	... 0	... 0	... 0
Hours at 37 C. ½	B +	B +	B +	rb +	B +1	... 0	B +1	... 0	rb +	... 0	rb ±	... 0	... 0	... 0
Hours at 37 C. 1	B +1	B +1	B ++	B +	B ++	rb Trace	B ++	... 0	B ++	... 0	B +	... 0	rb Trace	... 0
Hours at 37 C. 2	B ++	B ++	B ++	B ++	B ++	B +	B ++	... 0	B ++	... ...	B +1	... 0	B +	... 0
Peroxide test after aeration for 5 minutes	0		0		0		0		0		0		0	
1 hour	+		+		0		0		0		0		0	
2 hours	2+		+		±		0		0		0		0	

Symbols for methemoglobin reactions as in table 1.

Peroxide determined by the benzidine reaction. 0 indicates no reaction: ± very faint evidence of blue color at edge of potato; +, 2+, 3+, 4+ indicates increasing intensity of blue color of potato and surrounding fluid.

After four hours incubation the reactions of the X and Y cultures tested with washed red blood cells were quite similar. The change in color of the cells to reddish brown appeared first in the tubes containing the addition of sterile broth; at the end of one hour the methemoglobin reactions in all tubes were positive. Tests carried out with eight hour old cultures showed the first evidences of divergence in the action of the two varieties. At this time the browning of the cells by the X cultures was slightly delayed in the presence of broth (this phenomenon will be discussed later); while the Y strains exhibited signs of inhibition or inability to form methemoglobin unless enhanced by the addition of broth. This failure on the part of the Y cultures was not complete until the 18th hour of growth. The behavior of the X cultures toward red blood cells remained substantially the same from the 8th to the 24th hour periods of incubation. At the end of the 72nd hour, the ability of both X and Y cultures to oxidize the hemoglobin to methemoglobin had decreased.

Peroxide Production.—The tests for the presence of peroxide at different growth periods showed that, at the end of four hours incubation, the action of the two varieties was quite comparable, both giving a positive peroxide reaction when exposed to the air in a shallow layer for one hour. At the end of 8 to 12 hours growth, the amount of peroxide produced by the Y strains had decreased while that of the X strains had greatly increased. By the 18th, 24th, 48th, and 72nd hours incubation every X strain reacted strongly with benzidine, some after five minutes shaking and others after one hour exposure in a shallow layer. At the later periods of growth, the Y strains all failed to give a positive reaction with the exception of one culture (24 hours old) showing a trace of peroxide after exposure for 12 hours.

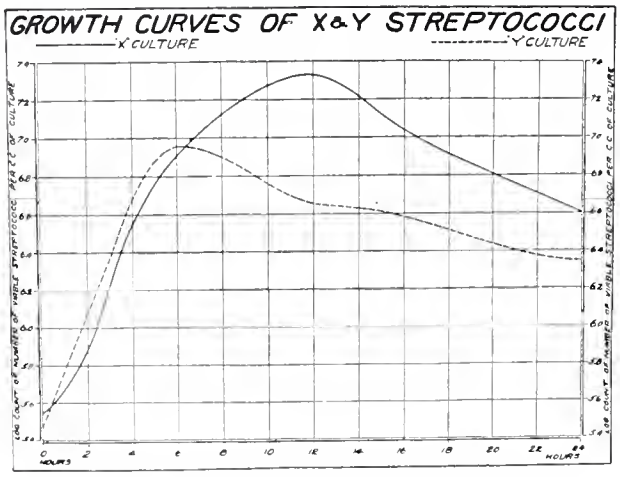
GROWTH CURVES OF X AND Y CULTURES IN BROTH

A comparison of the rate of growth of the two varieties was made to ascertain whether differences in the metabolic activity of the strains would throw light on their variable methemoglobin formation. Blake [5] in his study on the methemoglobin formation by streptococcus viridans concluded that the methemoglobin reaction "occurs only in the presence of living streptococci when they are able to carry on their metabolic activities."

In performing the experiment, two X and three Y cultures were grown in beef broth and after 2, 4, 6, 8, 12, 18, and 24 hours incubation,

definite dilutions were plated in blood agar, and the number of viable organisms per cc. at the different periods were estimated. The growth curves presented in the chart give the mean of the two X and of the three Y cultures tested.

The result of this experiment indicates some difference in the growth activity of the two varieties. The X strains reach the height of growth after 12 hours incubation, while the Y contain, apparently, largest number of viable organisms at the sixth hour. There may be minor deviations in the curve when different strains are used, but the principal character as illustrated remains the same. It seems reasonable to assume that the failure of the 18 hour cultures of the Y strains to act upon



hemoglobin, as compared with the positive results obtained with six hour cultures, is in some way related to a decrease or an inhibition of their metabolic activities. On the other hand, it should be emphasized that the ability of the X strains to form methemoglobin is not dependent upon any intensive multiplication since cultures tested during the fall of the growth curve and without any previous addition of nutriment give strongly positive reactions.

GROWTH IN BLOOD TUBES CORRELATED WITH METHEMOGLOBIN FORMATION

As further evidence that the failure of the Y strains to form methemoglobin when 0.5 cc. of an 18 hour culture was added to red blood cells could be correlated to a certain extent with the depression of metabolic activity, two X and two Y strains were tested comparatively for the amount of growth in the blood tubes at ¼, ½ and 1 hour

exposure in the water bath. Definite dilutions from the tubes to which broth and those to which salt had been added were plated in blood agar and the resulting colonies counted after 48 hours incubation. The two X strains tested showed evidence of multiplication in both "broth and salt" tubes at the end of ¼ hour. Plate counts of the Y cultures revealed, however, striking differences in the growth of organisms in the presence or absence of fresh broth. Definite increase in the number of organisms took place inside of 15 minutes in the water bath when a small amount of broth was added, while in the duplicate tube made up to an equal volume with salt solution, no increase in viable organisms was apparent even after one hour exposure.

Other accessory substances, such as serum (sheep), yeast extract,* 2% Witte peptone solution, and beef extract broth were tried in comparison with the beef infusion broth. The addition of each of these substances stimulated the Y cultures to form methemoglobin to some extent although none were equivalent to the beef infusion broth in this property. Serum and extract broth most nearly approached the beef infusion broth in action, while yeast extract and peptone water had much less influence.

ACID PRODUCTION

The final hydrogen ion concentration of ten X and ten Y cultures was determined colorimetrically after growth in beef broth and in 1% glucose broth for 24 and 68 hours. The X cultures grown for 24 hours in beef broth (P_H 7.2) fell between P_H 6.3 and 6.5, and the Y strains between P_H 6.5 and 6.8. When tested at the end of 68 hours growth in 1% glucose broth the acidity of the cultures ranged from P_H 4.4 to 4.7. Only one Y strain reached an acidity as low as the majority of the X strains but the differences were too slight to have any value in differentiating the varieties.

INFLUENCE OF ANAEROBIC VERSUS AEROBIC CONDITIONS

Since the accumulation of peroxide in cultures is dependent upon a free access of air, it seemed of interest to compare the behavior of the two varieties grown under anaerobic and aerobic conditions. Avery and Morgan [11] have shown that pneumococci grown anaerobically and subsequently kept in a reduced state, fail to produce peroxide. Furthermore, when pneumococci are exposed to a larger amount of molecular

* Yeast extract: 100 Gm. of brewer's yeast in 400 cc. of distilled water were adjusted to P_H 4.5 and boiled for ten minutes. After sedimentation the clear supernatant fluid was adjusted to P_H 7.4 before use.

oxygen during aerobic growth by having a broad surface area of broth, a greater amount of peroxide is formed than when grown in a deep tube of broth.

The data dealing with the aerobic and anaerobic cultures of X and Y strains are given in table 4. The methemoglobin reactions of X and Y cultures after 18 hours anaerobic growth (deep broth tubes with petrolatum seal) were almost identical. The addition of sterile broth enhanced the browning of the red blood cells by both X and Y cultures. As shown by the benzidine test the X cultures grown in this manner have been prevented from producing peroxide. When subsequently exposed to free access of air in a shallow layer for four hours, the X cultures, however, gave strong peroxide tests but weakly positive methemoglobin reactions. It was also noted that X cultures grown for 18-24 hours in a shallow layer of broth produced strongly positive peroxide tests and weak or negative methemoglobin reactions. The Y cultures, on the other hand, exhibited very slight differences, if any, in their action toward benzidine or on red blood cell suspensions whether grown for 18 hours anaerobically, aerobically in a deep broth tube or in a shallow layer of broth.

These observations on the behavior of X strains grown anaerobically and aerobically under different conditions of oxygen tension are analogous to observations of Neill and Avery [7] in regard to the action of reduced and oxidized pneumococcus extracts. As a result of extensive studies on the oxidation-reduction processes of pneumococcus extracts, these workers have drawn certain conclusions, namely: that the active system in the oxidation of hemoglobin to methemoglobin consists of two components, (1) the thermolabile component of the bacterial cell which is not removed by washing, and (2) the thermostable component which is present in meat infusion, yeast extract, etc. They [13] consider that the thermostable substances are the actual source of the oxidizing agents and that the thermolabile cellular constituent serves as a catalyst in accelerating the reactions involved. The loss in activity of active extracts on exposure to air is due to the inactivation of the thermolabile cellular component. This substance is susceptible to oxidizing agents and may be rendered inactive if exposed to peroxides or similar substances previous to their introduction into the oxyhemoglobin solutions.

The above considerations regarding pneumococcus extracts are given at some length because they seem to offer a logical explanation of the action of X streptococci which have been subjected to oxidation by

[13] J. Exper. Med., 1925, 41, p. 285.

TABLE 4

COMPARISON OF METHEMOGLOBIN REACTIONS OF X AND Y STREPTOCOCCI GROWN AEROBICALLY AND ANAEROBICALLY

Condition of Growth	X Streptococcus						Y Streptococcus					
	Growth in Test Tube				Growth in Shallow Layer		Growth in Test Tube				Growth in Shallow Layer	
	Anaerobic		Aerobic		Aerobic		Anaerobic		Aerobic		Aerobic	
18 hour broth culture	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Sterile broth	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...	0.1	...
Salt solution	...	0.1	...	0.1	...	0.1	...	0.1	..	0.1	...	0.1
2% sheep cells (all in c.c.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hours at 37 C. ¼	rb Trace	... 0	rb +	B +	... 0	... 0	rb ±	.. 0	rb +	... 0	... 0	... 0
Hours at 37 C. ½	B +-	rb +	B +1	B ++	rb Trace	rb +	B +	. 0	B +1	... 0	rb +	... 0
Hours at 37 C. 1	B ++	B ++	B ++	B ++	rb +	B +	B ++	.. 0	B ++	... 0	B +	... 0

Symbols as in table 1.

aeration. It is appreciated, however, that the analogy to a similarity of mechanism should not be drawn too closely, since in the experiments on streptococci up to date, living organisms have been present.

DELAYED REACTION OF 18 HOUR X CULTURES IN THE PRESENCE OF FRESH BROTH

The peculiar delay in the browning of red blood cell suspensions by X broth cultures in the presence of fresh broth has not been thoroughly worked out. From tables 3 and 4 it will be seen that peroxide production is in some way connected with this phenomenon since four hour broth cultures and those grown anaerobically, do not exhibit the retardation. The delay is more marked in the presence of sterile beef infusion broth than when other accessory substances such as extract broth, yeast extract, peptone solution are added. Furthermore, the delay is slightly greater when 0.3 cc. instead of 0.1 cc. of broth are added. A suggested explanation of this phenomenon is that this organism at the end of 18 hours growth, contains a complete system for oxidizing hemoglobin and is capable of acting almost immediately upon coming in contact with the suspension of red blood cells. When fresh broth is present during the test, the oxidizable substance in the broth is attacked before the hemoglobin and the characteristic browning is somewhat delayed.

ACTION OF SUPERNATANT FLUID OF BROTH CULTURES

The clear supernatant fluids of 5 and 18 hour X and Y broth cultures obtained by centrifugalization for 15 minutes were tested with benzidine and by adding to 2% sheep red blood cells. The peroxide tests were negative since only slight aeration had taken place. When incubated with red cell suspensions, weakly positive reactions appeared at the end of 1½ to 2 hours. The presence of a few viable organisms in this material as shown by the sterility test can account for this result. On the other hand, sterile filtrates of X and Y cultures were uniformly negative on incubation with the hemoglobin solutions containing blood catalase.

The supernatant fluid of two 18 hour X cultures which were completely aerated before centrifugalization, gave strong reactions with benzidine but had no effect on the red cell suspensions after two hours in the water bath. Since the cultures had been fully oxidized, no reaction could be expected.

Cultures tested after heating at 65 C. for 10 minutes, and at 55 C. for 30 minutes invariably were without effect on the blood cells. When

heated at 55 C. for 15 minutes, however, and sub-cultures demontrated viable bacteria, delayed but positive reactions in the blood tubes appeared.

ACTION OF SALT SOLUTION SUSPENSIONS OF WASHED COCCI

Blake,[5] in his study of methemoglobin formation by streptococcus viridans tested the salt solution suspension of the washed organisms from an 18 hour ascitic broth culture and found that methemoglobin formation took place only when nutriment material such as broth, ascitic fluid, peptone solution, or glucose was added. A similar experiment was performed with the X and Y green streptococci. Various accessory substances were added to the salt solutions suspensions of three X and three Y strains. Eighteen hour cultures in bottles of broth were centrifugalized, washed twice with salt solution and made up in one half the volume of 0.9% salt solution. The data are summarized in table 5.

It was immediately evident that the salt solution suspensions of washed streptococci are unable to attack red blood cells confirming the observation of Blake. In the presence of accessory substances such as beef broth P_H 7.4 and 6.4, extract broth, yeast extract, 2% peptone solution, 1% glucose solution and sheep serum, the sedimented cocci from the X variety were active in transforming the hemoglobin to methemoglobin. Slight differences in the reactions when the various substances were added may be noted in the table; for instance when peptone solution or yeast extract were added the reaction proceeded more slowly than in the presence of beef infusion broth and 1% glucose solution. The sterile filtrates of X and Y broth cultures were fully capable of completing the methemoglobin-forming property of the X cocci.

Methemoglobin formation by the sedimented and washed organisms from 18 hour Y strains took place in the presence of beef infusion broth and 1% glucose solution, but to a lesser degree with extract broth, peptone solution, serum or yeast extract. However, the addition of the sterile filtrates of 18 hour X and Y cultures to the salt solution suspension of Y strains failed to bring about the transformation of the blood pigments.

It is therefore evident that the salt solution suspensions of washed X and Y streptococci differ in their ability to form methemoglobin. Although the X cocci are quantitatively more active than the Y in the

TABLE 5

METHEMOGLOBIN REACTIONS OF SALT SOLUTION SUSPENSIONS OF X AND Y STREPTOCOCCI

	X										Y									
Suspension of washed streptococci	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Beef infusion broth Pн 7.4	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...
Beef infusion broth Pн 6.4	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...
Extract broth	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...
1% glucose solution	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...
2% peptone solution	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...
Yeast extract	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...
Serum (sheep)	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...	...
Filtrate* X 18 hour culture	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...	...
Filtrate Y 18 hour culture	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2	...
Salt solution	...	...	...	...	...	...	...	...	...	0.2	...	...	...	...	...	...	...	...	...	0.2
2% sheep red blood cells (all in c.c.)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Hours at 37 C. ¼	rb +	rb +	rb ±	rb +	... 0	... 0	rb ±	rb +	rb +	... 0	rb ±	rb Trace	rb Trace	rb ±	... 0	... 0	... 0	... 0	... 0	... 0
Hours at 37 C. ½	B +1	B +1	B +	B ++	... 0	rb Trace	B +	B +1	B ++	... 0	B +	rb +	rb +	B +	... 0	... 0	rb +	... 0	... 0	... 0
Hours at 37 C. 2	B ++	B ++	B ++	B ++	B +	B +	B +1	B ++	B ++	... 0	B ++	B ++	B ++	B ++	rb +	rb +	B +	... 0	... 0	... 0

Symbols as in preceding tables.

* The filtrate of the X culture gave a positive peroxide reaction. Control tubes of 0.5 cc. of the various accessory substances gave negative reactions with the red blood cell suspension.

presence of the various accessory substances such as beef infusion, serum, glucose solution, etc., a marked difference between the two is noted when the filtrates from 18 hour X and Y cultures are added. The presence of these filtrates completes the methemoglobin forming system of the X strains but not that of the Y.

The possible relation of these observations to the publications of Avery, Neill and Morgan,[7, 8, 11, 13] on the oxidation-reduction systems of pneumococci raises many questions which are not in the scope of this paper. These workers have shown that broth extracts of pneumococci contain a complete system for oxidizing hemoglobin to methemoglobin while phosphate suspensions of washed pneumococci contain an incomplete system and are inactive unless mixed with sterile broth, yeast extract, etc. The failure of sterile phosphate solution extracts to oxidize hemoglobin is claimed by Neill and Avery[13] to be due to the absence of the thermostable constituent, i. e., of auto-oxidizable or easily oxidizable substance which is removed by washing bacteria and which is present in heated extracts of animal and plant tissues.

Applying their deductions to the data reported in the preceding paragraphs, it is reasonable to assume that the X green streptococci in salt solution suspensions require only a small amount of oxidizable substance to complete the reaction, in fact 0.02 cc. of sterile broth or the filtrates from X or Y cultures are sufficient. For the completion of the methemoglobin-forming system of washed Y cultures a large amount of highly oxidizable or growth stimulating substance is absolutely necessary.

CORRELATION WITH PREVIOUS REPORTS ON METHEMOGLOBIN FORMATION BY GREEN STREPTOCOCCI

Reference has previously been made to the study of the methemoglobin formation of green (viridans) streptococci by Blake.[5] He tested the action of a large number of strains upon 5% washed sheep cells, and states that "no significant difference in the action of the different strains has occurred," although "there was considerable variation in the ability of various strains to form methemoglobin." With some strains only 0.1 cc. of broth culture was necessary for a positive reaction at the end of one hour in the water bath at 37 C., while with others 1.0 cc. was required. A careful perusal of Blake's work showed that his large series of comparative tests were performed with 12 hour ascitic broth cultures. This is a significant point and may explain his failure to note greater dissimilarity among the strains, since the Y streptococci as

described in this paper, frequently show a slight methemoglobin reaction after 12 hours growth but are negative by the 18th hour. Furthermore, the use of ascitic broth as the medium of growth may also tend to mask the difference between the X and Y varieties. In an experiment to verify this a Y culture was tested with red blood cell suspension after growth in serum broth for 18 hours. It gave a weakly positive methemoglobin reaction at the end of one hour in the water bath when .05 cc. was added to the blood cells as compared with the negative result obtained with 0.5 cc. of an 18 hour beef broth culture of the same organism. The presence of the small amount of serum (or ascitic fluid) may have stimulated the growth of the organisms in the blood tube.

In order to determine the optimum period of growth for methemoglobin formation, Blake studied the action of a broth culture of streptococcus viridans on red blood cell suspension after incubation for varying intervals from 3 to 60 hours. In this experiment, a positive methemoglobin reaction appeared at the sixth hour of growth and began to show a decline at the 18th hour. In the light of the action of the X and Y streptococci, Blake was apparently dealing with an X type in this experiment.

Lyall [4] studied the action of broth cultures of hemolytic and green streptococci on washed sheep cells and concludes that "some indifferent streptococci are green on the blood plate." He drew this conclusion from negative results obtained when 0.5 cc. of 18 hour calcium carbonate ascitic broth cultures were incubated for two hours at 37 C. in a water bath with 1 cc. of 5% sheep cells. It seems quite probable that these strains belonged to the Y variety. True gamma strains as described by Brown [1] are colorless when observed on or in blood agar plates.

AGGLUTINATIVE AND FERMENTATIVE DIFFERENCES

In order to prove that the differences in the ability of green streptococci to produce peroxide was not merely a functional variation among antigenically similar strains, immune serums for three X and three Y strains from separate cases were prepared. Direct agglutination tests of the X and Y cultures from each of these cases showed no cross reactions between the X and Y strains. Nor has any evidence of similarity been noted up to date between the X or the Y strains from different cases.

The X and Y strains from an individual case also differed from each other in their ability to ferment the carbohydrates, such as lactose, salicin, mannite, raffinose, and inulin. Fermentation tests brought out a marked similarity in the reactions of the majority of Y strains; all

except two fermenting lactose and salicin, and failing to attack mannite, raffinose and inulin (streptococcus mitis according to Brown's [1] classification). The X cultures tested belonged to many fermentative groups.

Whether this distinction between green streptococci based on the marked difference in their ability to produce peroxide has any pathologic significance will bear further study. Both X and Y varieties have been recovered in pure culture from the heart blood and from the joints of rabbits inoculated with tonsil material in a study of focal infections. Two strains isolated recently from blood cultures in cases of endocarditis proved to be strong peroxide producers, the X type. Blake [5] attempted to correlate strong methemoglobin formers with the source and virulence of the cultures. He concludes "that a study of the results shows no relation between the source and virulence of the strains and their methemoglobin titers." As noted earlier, he used 12 hour ascitic broth cultures in testing the cultures and under these conditions the difference between the varieties would tend to be masked to a certain degree.

Among the 35 strains studied, the differences between the two varieties has been sharply defined when actively growing cultures have been compared. It should be noted, however, that the growth activity of the individual strain is of considerable importance in testing its peroxide production and methemoglobin formation. For instance, a Y culture which has grown poorly by the 18th hour may not exhibit the complete failure to form methemoglobin in the absence of fresh broth which is evident when the culture is incubated four hours longer; whereas X cultures under unfavorable growth conditions may not show the delayed reaction with red blood cells when broth is added.

The green streptococci designated as X and Y have remained true to the described characteristics in numerous tests carried out over a period of six months; no fundamental variations in the peroxide production or methemoglobin formation of the strains tested under relatively similar conditions has been observed. A study of a larger series, however, may reveal the presence of intermediary strains between the varieties described.

As an interesting contribution to this work, it should be noted that the X green streptococci are more closely related to the pneumococcus than are the Y strains. Although the peroxide production of three pneumococcus cultures tested was more vigorous than observed for any culture of streptococcus, the time of its appearance and the conditions under which it could be demonstrated in living cultures were materially

the same. Associated with the similarity in peroxide production, other similarities such as the yellowing of chocolate agar, the ability to oxidize hemoglobin to methemoglobin without any additional substance, and even the slightly delayed reaction in the presence of fresh broth as described for the X strains were demonstrable. A study of sterile extracts prepared for the X strains may yield further evidence of a similarity between this streptococcus and pneumococcus in respect to their oxidation-reduction systems. It should be emphasized that none of the streptococci was soluble in bile.

DISCUSSION

The observations on the differences in the peroxide producing and the methemoglobin-forming properties of green streptococci are reported as a possible basis of differentiation without attempting to work out fully the biological or chemical nature of their action. The experiments to date seem to indicate that a difference in ability to produce peroxide is the primary factor on which other observed variations such as, the appearance on surface blood plates, on chocolate agar slants and the formation of methemoglobin in the test tube depend. It is also evident that the diversity in the action of 18 hour cultures of the two varieties on red blood cell suspensions may be correlated, to some degree at least, with differences in growth activity of the strains. Whether the cessation of growth of the Y strains is due to a greater sensitivity of this organism to the products of growth, whether there is some difference in the enzymes elaborated by the two varieties, or whether one is dealing with a fundamental difference in the oxidation-reduction systems will require further study. Sterile extracts of the two types of streptococci should be prepared to determine whether one or both possess active oxidation-reduction systems in the absence of living organisms. A study of sterile filtrates of streptococci grown and kept under different conditions of oxygen tension may also be of value. Furthermore, the use of crystalline (catalase free) hemoglobin solutions in comparison with hemoglobin solutions containing blood catalase is essential, since it has been shown [7] that under certain conditions the presence of blood catalase protects the hemoglobin from oxidation.

CONCLUSIONS

Green (alpha) streptococci may be differentiated into two varieties based on peroxide production and methemoglobin formation of 18-24 hour broth cultures. The X variety gives a positive peroxide reaction

with benzidine and a strong methemoglobin reaction when added to washed sheep red blood cells in the test tube; the Y variety at that period is negative for peroxide and oxidizes hemoglobin to methemoglobin only after the addition of sterile broth, serum, etc.

Four hour cultures of the two varieties are found to be alike in peroxide and methemoglobin production but after an incubation of 18-24 hours at 37 C. a difference in action is apparent.

The growth activity of the two varieties is shown to be different. The X strains reach the height of the growth curve after 12-14 hours incubation, while active multiplication of the Y strains drops decidedly after 6 hours.

Difference in the methemoglobin reaction of the X and Y strains is evident when salt solution suspensions of washed organisms are tested. To complete the reaction of the salt suspensions of X cultures only a small amount of accessory or oxidizable substance is required, while the Y salt suspensions are active only in the presence of a highly oxidizable or growth stimulating substance. Sterile filtrates of X or Y broth cultures complete the methemoglobin reaction of X cocci suspended in salt solution but fail to activate the Y cocci.

The X streptococci more closely approach pneumococci in their peroxide-producing and methemoglobin-forming properties.

Certain possible correlations with the work on the oxidation-reduction systems of the pneumococcus are discussed.

RÔLE OF BACTERIOPHAGE IN STREPTOCOCCUS INFECTIONS

AN INTERPRETATION OF CERTAIN CULTURAL CHARACTERISTICS

ONE PLATE

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With the accumulation of more and more experimental work regarding the bacteriophage, the statement of d'Herelle that transmissible lysis is but a single phenomenon in a very complex series of changes, is becoming increasingly significant. Various observers have detailed observations that can be readily explained by broadening the field included in the term bacteriophagy. Indeed, this is but logical, since regardless of the nature of the bacteriophage itself, it depends on the reaction of living organisms for its manifestations. It would be unusual for any activity of life, either on the part of the bacteriophage or the bacterium, to result in events so narrowly limited as the classical phenomena are. If we assume that the bacteriophage is a living ultramicrobe, then the expectation of variation in the result of action and interaction between it and the bacterium would be greatly broadened. Hadley [1] has ably advanced this conception of bacteriophagy; and has reported observations that indicate a diversity of activity, sufficiently distinct from the classical transmissible lysis, to open new lines of approach for the study of this very interesting and significant question.

In a previous article I [2] have reported a series of studies on the action of the bacteriophage in streptococcus infections. The detailed experimental work done in this connection has given the clue to a series of studies on cultures of streptococci, which have led to very significant results.

For studies of this character a medium was desired for the cultivation of the organisms that would allow the bacteriophage to manifest its greatest activity. To this end a simple nutrient agar without any sugar was selected, with the P_H adjusted to 8. For the fluid cultures a broth of the same composition and reaction was used. It has been found that such a medium is easily prepared by the use of Difco dehydrated agar and broth, using a weak veal infusion for a

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[1] J. Infect. Dis., 1925, 37, p. 35.
[2] J. Lab. & Clin. Med., 1926, 11 (in press).

solvent instead of water, and adjusting the reaction to the desired P_H. In the case of the broth it is necessary to add the usual amount of sodium chloride. The veal infusion is readily obtained by shaking 10 gms. of Difco dehydrated veal with one liter of water and allowing to remain in the ice box over night. To obtain clear and transparent medium in this manner it is necessary to bring the broth to a boil and cool after the reaction has been corrected and before it is filtered. It is preferable to clear the agar with egg albumin after the reaction has been corrected.

In regard to the technic of obtaining transmissible lysis it has been found that very young cultures of streptococci must be used, and it is sometimes necessary to plant these cultures from broth tubes containing not older than six hours growth.

Needless to say that the lysis of streptococcus cultures is not nearly so striking as the lysis of dysentery or typhoid organisms. It is sometimes difficult to recognize the occurrence of lysis as in many strains the occurrence of secondary cultures follows so rapidly that it is sometimes confused with no lysis at all.

There have been few references in the literature to phagic activity against the streptococci. Experiments attempting to adapt a bacteriophage active against the gram negative rods to the gram positive cocci have been in the main unsuccessful. To arrive at some explanation of this fact, as well as to isolate a strain of streptococcus which would prove susceptible to the bacteriophage, a large number of strains have been studied.

On careful inspection of these strains with reference to the bacteriophage they are divisible into a number of types that are more or less distinct. The studies have all been done on freshly isolated strains. Stock strains are not useful in this connection for reasons that will be developed. The following scheme of classification has been devised to facilitate description only, and does not account for all of the strains studied. Most of them can be placed in it, however.

1. Strains growing on the isolation culture medium, but failing to grow subsequently, on agar and in broth, type 1; those failing to grow in broth, but growing on agar, type 2.

2. Strains that grow readily in broth and on agar: a. Strains showing regular normal colonies and giving transmissible lysis, type 3; those failing to give transmissible lysis, type 4: b. Strains giving irregular and proliferating colonies on agar; with colonies normal after 24 hours incubation but becoming eroded and irregular after varying periods, giving transmissible lysis, type 5; with colonies large and flat after 24 hours but subsequently developing secondary normal colonies, not giving transmissible lysis, type 6.

TYPE 1. STRAINS GROWING ON THE INITIAL ISOLATION CULTURE MEDIUM BUT FAILING TO GROW ON TRANSPLANTS

Cultures of this type are relatively rare. Only one such has been encountered in the course of these studies. It was isolated from a pathologic condition on dextrose blood agar. At the end of 24 hours the colonies were larger than normal and more elevated. After 48 hours the colonies began to flatten, and the central portion became transparent. In the course of five days the colonies were thin films, barely detectable on the medium. Transplants made at various intervals onto a variety of mediums failed to show growth at any period.

Smears of this culture revealed typical streptococci at the end of 24 hours with the exception that all of the cells failed to retain the gram stain. After 48 hours the smears failed to show organisms at all, although there were still seen grotesque forms that might or might not have been atypical cells.

This series of observations was interpreted as indicating that the original culture contained a bacteriophage of such virulence that the organism was completely overcome. Of course the test of transmissible lysis was not secured, but enough other evidence of bacteriophagic activity was observed to be fairly convincing that the changes were the result of true bacteriophagic activity. There was an initial period of proliferative growth as evidenced by the large colonies, a change in the bacterial cells producing poor staining reactions, and final sterilization and destruction of the growth. Of course, this series of events could take place in the normal course of growth and death of a bacterial culture, but it is inconceivable that they should happen in such a short period of time.

Unfortunately the patient from which this culture was obtained left the hospital before more material could be collected. It is possible that an active bacteriophage could have been isolated from the pus of the lesion.

TYPE 2. STRAINS GROWING IN THE INITIAL CULTURE, GROWING ON AGAR TRANSPLANTS, BUT FAILING TO GROW IN TRANSPLANTS MADE INTO BROTH

Several strains have been isolated that fall into this classification. The most typical example was isolated from the blood stream and was of the viridans type. Ten cc. of blood introduced into 300 cc. of dextrose broth showed after 18 hours incubation, a definite but slight cloud. Smears from this culture showed a streptococcus staining poorly. After 36 hours incubation, smears from the culture failed to show organisms. Transplants made at the 18 hour stage showed a growth of essentially the same character as described in the previous type, except that the stages were prolonged. Transplants subsequent to 18 hours were consistently sterile. The growths on agar transplants were constantly of the same character, the number of gram negative cells increasing with age, and requiring about 12 days to reach the end stage of lysis.

A very striking phenomena could be produced with this organism. An agar slant heavily seeded with the strain, after incubation for 24 hours, was washed into a tube of broth. This produced a moderate

cloud. The broth culture thus secured showed, after 12 hours incubation, markedly more turbidity than at the beginning. After a further incubation of 18 to 20 hours the broth became perfectly clear and remained sterile indefinitely. An occasional tube would show a secondary growth after a week or more at room temperature. This sequence of events could be induced constantly as described; until, with a constant increase of virulence of the bacteriophage, the time which the culture remained viable on agar became less and less, and finally after about a hundred transplants the organism was completely overcome, subsequent inoculations remaining sterile.

During the time that this culture was under observation other strains of streptococci were subjected to the action of the phage in the lysed cultures. In this series of experiments 22 strains of streptococci were used, 14 from normal throats and 8 from pathologic sources. Only one of these was lysed by the phage. It was a strain of hemolytic type isolated from the blood stream and gave complete lysis on the eighth passage. The details of this experiment and the clinical results obtained by the use of the bacteriophage therapeutically on the patient from which the strain was isolated are given in a previous paper.[2]

TYPE 3. STRAINS THAT GROW READILY ON AGAR AND IN BROTH, AND THAT GIVE TRANSMISSIBLE LYSIS, COLONIES REGULAR

There has been encountered but one strain that falls into this classification. It is the strain mentioned above which gave transmissible lysis in the experiment with the filtrates of the spontaneously lysing strain. An hemolytic streptococcus isolated from the blood stream, it was subjected to the action of the phage as soon as broth cultures could be obtained. On the eighth passage it was completely lysed and continued to be lysed on subsequent passages, although secondary growths in the lysed tubes were the rule. At all times on agar the colonies were normal in appearance, and smears from them revealed organisms that retained the gram stain in practically every individual.

This strain is interpreted as being a sensitive strain, not mixed with the bacteriophage.

TYPE 4. STRAINS GROWING NORMALLY ON AGAR AND IN BROTH, FAIL TO GIVE TRANSMISSIBLE LYSIS, COLONIES REGULAR

A number of strains have been isolated that belong to this general type. The colonies on agar appear normal, and stained smears show normal gram positive cocci. Repeated attempts to produce lysis by

phages from a number of sources have proven failures. Strains belonging to this type are considered as being completely resistant to the action of the bacteriophage, probably by virtue of having come in contact with a strong phage in nature and having developed a complete resistance to the lytic action. There is experimental evidence to support this conclusion that will be made clear in a consideration of the remaining types to be discussed.

TYPE 5. STRAINS WHICH GIVE VARIABLE GROWTH ON AGAR AND IN BROTH, GIVE TRANSMISSIBLE LYSIS, COLONIES FIRST REGULAR, SUBSEQUENTLY BECOMING IRREGULAR AND PROLIFERATING

Strains of this type seem to be fairly common. Broth cultures of such organisms are sometimes completely lysed, but more often partly lysed by the bacteriophage. These strains exhibit other phenomena that are more interesting and significant than transmissible lysis. The characteristic behavior of such cultures is first the production of normal appearing colonies, containing only gram positive cocci; and, secondly, an irregularity appearing as an erosion of the existing normal colony, at which time the gram reaction indicates considerable numbers of individuals negative. The time elapsing in each of these stages is variable according to the strain but seems to be constant in the same strain. As a rule, the end result of these cultures on agar is a definitely irregular colony containing a mixture of gram negative and gram positive individuals in varying proportions. The number of gram negative individuals seems to parallel roughly the sensitiveness of the strain to the bacteriophage.

A clue to the significance of these changes was obtained when the strains were studied in broth cultures. After 24 hours incubation, smears from the cultures showed only gram positive cocci. Subsequently the same changes in staining reaction as was observed on agar was obtained. If, however, the tube was allowed to remain from one to two weeks at incubator or room temperature, smears showed that the gram negative organisms had disappeared, and that only the gram positive cells remained. It has been interpreted that the gram negative cells disappear by virtue of lysis. Transplants from the old broth tubes at the time when gram positive cells only were found gave rise to colonies on agar that remained normal in appearance, and in general showed only gram positive individuals. Attempts at transmissible lysis with cultures thus secured were unsuccessful. These findings probably indicate the mechanism by which strains of type 4 naturally occur.

Various strains that were placed under this classification gave variation in some of the details noted above. Some of the strains after several transplants would change to the type to be described under the next head. Other strains would show at the end of 12 hour incubation normal colony form, but smears would show even at that early stage numerous gram negative individuals. After 24 hours incubation smears made from the colonies showed many of the gram negative cells to be spherical in shape and of large size. Many of these cells were several times the diameter of the normal coccus. Such a colony, if studied through the low power objective, was seen to be composed of an outer zone of thin growth in which the cells were normal; the entire inner portion of the colony being composed of large cells, whose size and number were even more striking when examined in this manner than in stained smears.

Some of these colonies were examined over a period by placing a sterile cover glass over a growing colony, and observing a single field under the oil immersion objective. The set-up was not disturbed, and it was thus possible to examine the same cell over any desired period. It was definitely established in this manner; that the large cells developed from normal cells, that they occurred singly, in short chains, as terminal cells on the end of normal chains, and in the middle of normal chains. It was further established, that as long as the large cells were capable of division it may give rise to a second large cell, or a normal cell, according to no observed order. A few of these cells were seen to disappear; and it is thought that the observations warrant the conclusion that the large cells, in all probability, represent a stage of lysis. It is possible that such strains by some means have delayed the normal course of lysis, so that a stage, usually of short duration, may represent a large proportion of the actual lysis.

Several strains isolated giving the above characters are all from pathologic sources. Many of the other strains studied showed at various stages a few of these large cells, but the phenomena was much less striking than the typical. The ultimate fate of such cultures after a varying number of transplants, was the gradual decrease in the number of large cells, and the final result of complete resistance to phagic activity. A single strain, however, reacted in the opposite manner, and eventually failed to give growth in transplants. Filtrates made of the broth cultures of such strains exhibited a weak lytic action on sensitive strains of other types.

Although the occurrence of transmissible lysis was given as a part of the criteria for this type, these last described strains do not fulfill it. This was due to the peculiar manner of the growth in broth. Immediately after isolation the broth cultures were heavy, but with each successive transplant the growth was less, until there was none. It is curious that these strains showed this outcome in broth while at the same time on agar the bacterium was able to react in the opposite manner and produce resistant colonies.

A possible explanation of these strains is that they are mixed with the bacteriophage, and that the balance existing between the lysin of the phage and the antilysin of the bacterium is either at an equilibrium, or being displaced, either in the direction of the lysin or the antilysin, depending on the environment.

TYPE 6. STRAINS THAT PRODUCE IRREGULAR AND PROLIFERATING COLONIES, WHICH AT FIRST ARE IRREGULAR AND TRANSPARENT, BUT SUBSEQUENTLY SHOW THE PRESENCE OF SECONDARY RESISTANT COLONIES, AND NEVER GIVE TRANSMISSIBLE LYSIS

Strains of this type are fairly common. Most of them have been isolated from normal throats and from tonsils removed at operation. The character of the colony can be recognized with the low power hand lens, but the features are more striking when viewed through the 16 mm. objective. The appearance of these colonies is quite characteristic. The outline is irregular and the border is as elevated, sometimes more so, than the center. There is a transparency that is quite in contrast with the opacity of normal colonies. The mean diameter of these colonies is many times the diameter of normal colonies and may reach 3 or 4 mm.

After 48 hours incubation there appears usually at the periphery, but sometimes in the center, of the colony a small, round, elevated, opaque sub-colony. Sometimes these appear only after prolonged incubation, sometimes there will be only one sub-colony to several original colonies, and at other times every original colony will develop one or more sub-colonies. By using a fine platinum needle and manipulating it under a low power magnification, transplants can be made from portions of these colonies that do not show secondary colonies. Such transplants remain sterile. Smears from these areas show almost 100% gram negative cocci. In the same manner transplants from the sub-colonies always grow, but instead of giving colonies of the forms just described, produce small, circular, elevated, opaque colonies. Transplants made from a mixture of the two areas produce colonies that are

true to the parent type. Some resistant strains isolated in this manner have remained purely resistant as long as kept under observation, thus becoming characteristic of type 4 strains. Others, after a few transplants, gradually revert to the original type.

Attempts at transmissible lysis have failed with this type of organism. Certain strains of other types which were showing transmissible lysis, suddenly developing characteristics of this group, simultaneously show a loss of susceptibility to transmissible lysis. A possible interpretation of the observed facts exhibited by this group is that the strains are mixed with a bacteriophage, which has a marked ability to sterilize a large part of the culture, but certain resistant organisms remaining viable, give rise to resistant sub-colonies in spite of the phagic activity.

TABLE 1

TYPES OF STREPTOCOCCI IN RELATION TO THE BACTERIOPHAGE

Type	Initial Culture	Subsequent Culture			Interpretation
		Agar	Broth	Lysis	
1	Lysis*	None	None	Negative	Sensitive
2	Lysis*	Lysing	None	Negative	Sensitive, mixed strain
3	Normal	Normal	Normal	Positive	Sensitive, unmixed strain
4	Normal	Normal	Normal	Negative	Resistant
5	Irregular, sensitive	Irregular, sensitive	Normal	Positive	Sensitive, mixed strain
6	Irregular, resistant	Irregular, resistant	Normal	Negative	Resistant, mixed strain

* Loss of gram staining property of cells cultivated on alkaline agar.

Table 1 gives the data relative to the various types described in simplified form. The types of cultures described above are the most outstanding variations from the normal that have been observed. Needless to say that in a survey of many strains, transition stages have been observed that defy classification, and many changes from one type to another as was described have been seen. As a matter of fact, the ultimate end form of those cultures that do not succumb to the action of the bacteriophage has been a resistant strain giving normal colonies, so far as form is concerned. The changes observed were more marked immediately after isolation, and only on the medium described. On any medium that allowed a shift of the P_H to less than 7, practically all strains uniformly gave colonies of normal form, and smears gave 100% gram positive cocci. Gelatin added to the fluid media would likewise inhibit the appearance of all the phenomena observed. An occasional strain exhibited the phenomenon on carbo-hydrate media. Such strains,

although fermenting the sugar to a slight degree failed to convert the P_H to lower than 7.

In the instances where transmissible lysis was obtained the source of the bacteriophage was some other strain of streptococcus that exhibited marked spontaneous lytic activity. By using only material from freshly isolated streptococcus cultures it was hoped that an erroneous viewpoint, in terms of resistance to disease, due to the use of a phage isolated from the intestine and adapted to the streptococci, might be avoided; as such a course would not fulfil the requirements of natural infection and resistance.

It seems clear from the observed data that, except in the case of type 3, we are dealing with mixed cultures in the sense used by d'Herelle. To account for all the facts, it seems necessary that we admit into the realm of bacteriophagic activity, other phenomena than transmissible lysis. This has already ample support from other workers. The poor staining of the cells, the swelling and disappearance of these cells, the development of resistant cultures, the rapid proliferation, the transparency of affected colonies, the spontaneous lysis of fluid cultures, the unusual form of the colonies, and the inhibition of these phenomena by acid ions and gelatin, are all in agreement with the conception of bacteriophagic activity.

Without committing myself to the belief in the living nature of the bacteriophage, d'Herelle's theory seems to explain all the facts observed. He has, by advancing the parasitic nature of the phage, given us the mechanism by which many phenomena may be interpreted. There is nothing more variable in nature than the result of the action and interaction between host and parasite. It is reasonable then to expect that if such parasitism occurs the effects on the parasitized bacterium would be variable. It seems to be necessary for mixed cultures to exist in nature that the bacteriophage might be conserved. It is obvious that if the invariable result of parasitism of the bacterium is death of the strain, and if the phage is an obligatory parasite, the life of the phage would be self-limited. This is of course contrary to the observed facts, as well as contrary to the general laws of nature. There is nothing revolutionary then in the conception, that many strains of bacteria may exist in a state of mixture with the bacteriophage without being destroyed, and that cultures of such strains should exhibit markedly various changes. d'Herelle and others have made it clear that such cultures do occur; but without emphasizing the possibility of their frequency, and the extreme variation

in their form, nor has their significance with reference to natural infection and resistance been extensively studied.[3]

Many authors have fallen into the fallacy of drawing general conclusions from studies done on one or two strains. Such methods are obviously incomplete, and although they may be indicative of the possibilities of the action of the bacteriophage, they fail to give a comprehensive idea of the state of facts actually existing in nature.

The conclusions reached after a study along the lines indicated are; (1) that practically all strains of streptococci if studied on a suitable medium immediately after isolation are seen to be "mixed cultures" (Cowan,[4] working with laboratory strains of streptococci was able to dissociate all of them into "rough" and "smooth" or *R* and *S* types); (2) that the form of these cultures is extremely varied; and (3) that the behavior and ultimate fate of the strain can sometimes be predicted, but not always, by the colony form, the morphology and staining reaction of the cells.

With these cultural variations in mind, it becomes of interest to study streptococcus infections with a view to the distribution of various types of organisms. If we admit the role of the bacteriophage in the exogenous immunity of d'Herelle, then it would seem that there should be some logical relationship between the character of the infection produced by a strain of streptococcus and its relation to the bacteriophage.

We may conveniently divide streptococcus infections into three arbitrary groups: infections that are very mild, and recovery is the end result with simple or no treatment, group 1; infections that vary in severity, but recovery usually follows if no complicating pathology occurs, group 2; infections that end fatally in spite of every therapeutic means being employed, group 3.

Infections occuring in group 1 are undoubtedly very common. It is rare, however, that patients of this type are under observation, and still more rare that bacteriologic studies are done. A few cases only have been studied in detail that belong to this group.

Case 1.—A patient, following an operation for a mastoid infection suddenly developed septic symptoms. A blood culture taken at the initial peak of temperature revealed a streptococcus that fell into type 2, that is, it was sensitive to the action of the bacteriophage, and was a mixed strain. This patient recovered promptly without medication of any kind. This case with the accompanying experimental work is reported in detail in a previous article.[2]

Case 2.—A patient, following an operation for mastoid infection and developing septic symptoms was found to have a blood stream infection of the streptococcus that fell into type 5, that is, a strain that is mixed with the bacteriophage and was overcome by it in liquid cultures very

[3] In his last book, "The Bacteriophage and Its Behavior," 1926, which has appeared since this article has gone to press, d'Herelle has considered the question of mixed cultures more completely.

[4] Brit. J. Exper. Path., 1922, 3, p. 187; 1923, 4, p. 241.

slowly. In this instance, the recovery of the patient was complete and without medication, but the time required was longer than in case 1. A matter of two weeks elapsed after the initial blood culture before the temperature remained at normal. The blood stream was found to be sterile, however, on the third day after the initial culture.

Case 3.—A puerperal streptococcus septicemia due to a strain belonging to type 3 was treated by intravenous injections of three 1 cc. quantities of a lysed streptococcus broth culture. After 24 hours the blood stream was sterile, and the patient made a slow but uneventful recovery. Studies on the strain of organism isolated from this patient were done subsequently to the therapeutic test; and indicated that the bacterium was a normal strain, that is, it was sensitive to the bacteriophage but not mixed with it.

These three examples of prompt recovery from a streptococcus septicemia are thus definitely linked with the bacteriophage as a factor in the sterilization of the blood stream. Experiments reported elsewhere confirm this view. These examples are not the total number of spontaneous recoveries observed, and it is well to add that no spontaneous recovery has occured without essentially the same bacteriologic findings as given above, and all cases that have revealed such findings have been spontaneous recoveries.

Infections occurring in group 2, are relatively common and are chiefly of the local type. Studies on the bacteria isolated from such infections may reveal either sensitive or resistant organisms. All of them are mixed with the bacteriophage, and the end result of the strain varies accordingly as the artificial methods of cultivation have influenced the balance of lytic activity and resistance of the bacterium. One interesting case is that of a local infection, which had resisted medication of various kinds over a period of six weeks. A small quantity of bacteriophage was placed in the ulcer. The patient's temperature dropped rapidly to normal and the wound began to heal. Of course the fallacy of "post hoc propter hoc" is an easy one to fall into, but in this case experimental study proved the strain to be sensitive and unmixed before the application of the bacteriophage, while subsequently cultures isolated were sensitive and mixed.

Infections in group 3 are very common. There is little to say of them except that they are characteristic fatal streptococcus septicemia. The bacteriologic studies in such cases reveal always a bacterium of the resistant type. Most of them are of type 4, that is, so highly resistant that no evidence of phagic activity can be demonstrated. All such strains fail to give transmissible lysis when subjected to a strong bacteriophage. Most of the infections in this group have been subacute bacterial endocarditis due to streptococci of the viridans type. In every case observed attempts to use the bacteriophage as a therapeutic agent have proven futile.

With a survey of streptococcic infections we have seen that in no case of spontaneous recovery has a resistant bacterium been demon-

strated, and that in every case of fatal streptococcus septicemia a resistant bacterium has been isolated. Corresponding to those bacteria that are midway in their reaction to the bacteriophage, there are infections that are midway in their virulence. Thus we see a parallel between the virulence of the bacterium and virulence of the bacteriophage in inverse ratio.

Table 2 shows the chief features of a number of cases of streptococcus septicemia, illustrating the parallel between the outcome of the infection and the relationship existing between the bacterium and the bacteriophage.

There remains a word to be said about the strains of streptococci that were isolated from normal throats. About 50 such strains have been observed. In practically every instance it has been possible to detect morphologic and cultural variations thought to be the result of bacterio-

TABLE 2

THE RELATIONSHIP BETWEEN THE OUTCOME OF THE DISEASE AND THE SENSITIVENESS OF THE ORGANISM TO THE BACTERIOPHAGE

Case	Disease	Treatment with Bacteriophage	End Result	Organism	Type
1 to 5 inclusive	Endocarditis	Intravenously	Fatal	Resistant	4
6	Pyemia	Intravenously	Fatal	Resistant	4
7	Meningitis	None	Fatal	Resistant, mixed	5-6
8, 9, 10	Chronic septicemia	Intravenously	Slow recovery	Sensitive mixed	5
11	Puerperal septicemia	Intravenously	Rapid recovery	Normal	3
12, 13, 14	Acute septicemia	None	Rapid recovery	Sensitive, mixed	2

phagic activity, and indicating mixed strains. The types of these strains have varied, but rarely has a completely resistant strain been encountered. It is possible that the streptococci existing as mixed strains are the source of the bacteriophage which is called upon as a resistant factor in streptococcus septicemia.

The greater part of the work which has been done on the bacteriophage has dealt with bacteria that normally do not occur in the human body, and that in general, might be termed epidemic diseases. The streptococcus infections that have been studied in the present work are not epidemic diseases and organisms identical with virulent strains can readily be isolated from a normal throat. The evidence presented above seems to explain the mechanism by which these bacteria are carried normally, and by which they occasionally are capable of producing virulent and fatal disease.

If we admit the various phenomena that have been observed in so-called mixed cultures of streptococci to the realm of bacteriophagy, then it is possible to apply d'Herelle's theory of exogenous immunity to the explanation of observed facts concerning streptococcus infections, and the resistance to such infections with surprising completeness.

SUMMARY

Streptococci react in a characteristic manner when mixed with bacteriophage, some being resistant, others sensitive. This variability results in colony forms that are characteristic as well as in variation in regard to transmissible lysis. On the basis of such data the strains studied are divided into six types.

Type 1. Strains give growth on the isolation culture, colony form irregular with spontaneous lysis, no growth in subsequent transplants.

Type 2. Strains are essentially like type 1 except that transplants on agar grow, reproducing the characteristic colonies, while transplants in broth remain sterile.

Type 3. Strains have normal colonies, normal growth in all transplants, and are susceptible to the action of a lytic principle.

Type 4. Strains are like type 3, except that they are not susceptible to transmissible lysis.

Type 5. Strains have a characteristic sensitive type colony which is rough, and eroded; showing the phenomenon of transmissible lysis.

Type 6. Strains show resistant colonies which are rough and irregular, with small subcolonies developing in the original after a varying period of incubation; no transmissible lysis.

A possible explanation is that most strains of streptococci are mixed with bacteriophage; and that the colony forms are the result of the reaction of the bacterium to the phage, this ranging from complete sensitiveness to complete resistance.

Study of the distribution of the various types in streptococcus blood infections indicates that in those cases that recover, the strain is sensitive to bacteriophage and mixed with it. In cases that end fatally the strains isolated are resistant, and mostly unmixed with bacteriophage.

Studies of the normal flora of the throat indicate that the source of the bacteriophage in streptococcus blood infections probably is the normal throat streptococci, many strains of which are mixed with bacteriophage.

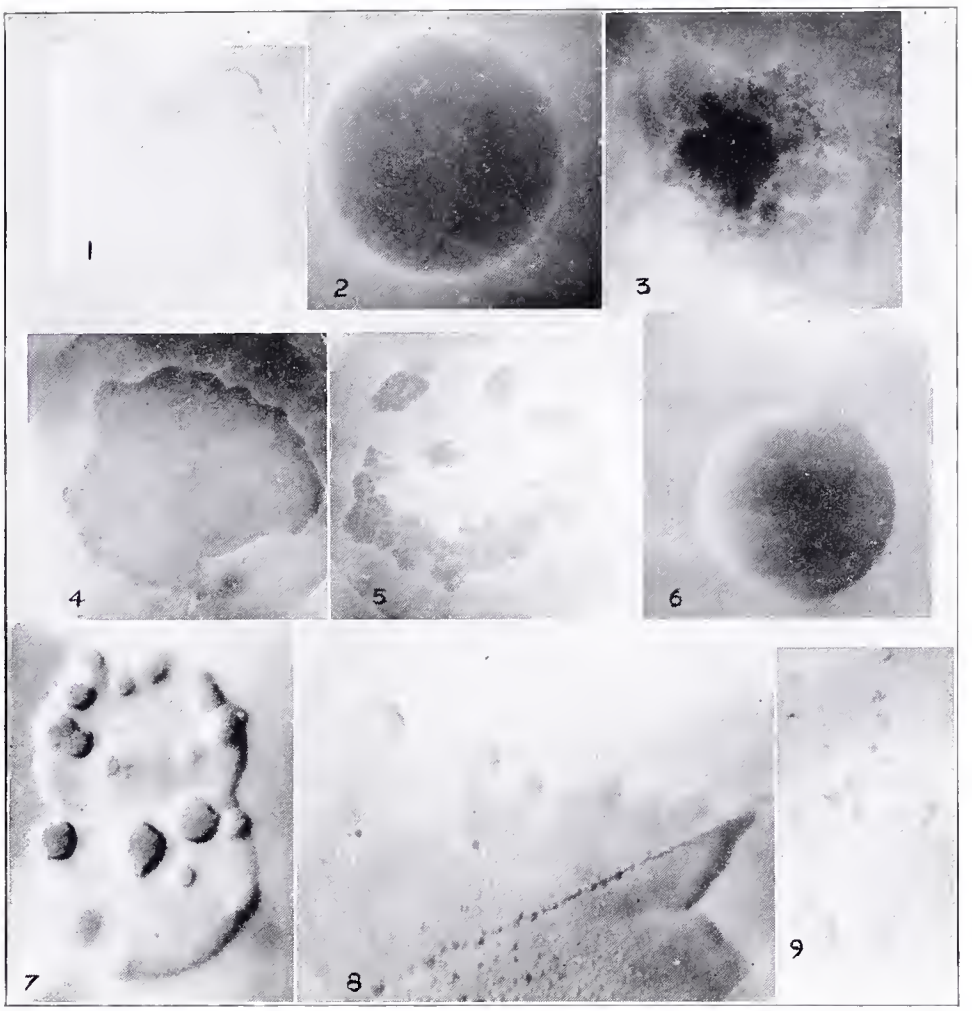

Fig. 1.—Barely visible remnants of a sensitive colony. Characteristic of type 1 and 2 strains. (× 100.)

Fig. 2.—Twenty-four hour colony of a sensitive mixed strain. (× 100.)

Fig. 3.—Same colony illustrated in figure 2 after 72 hours growth. Characteristic of type 5 strains. (× 100.)

Fig. 4.—Twenty-four hour colony of a resistant mixed strain (× 50.)

Fig. 5.—Same colony illustrated in figure 4 after 72 hours growth, showing secondary resistant subcolonies. (× 50.)

Fig. 6.—Twenty-four hour resistant mixed colony. (× 100.)

Fig. 7.—Fifteen day old colony from same plate as figure 6, showing less lysis than the strain illustrated in figures 4 and 5, but showing resistant subcolonies. (× 100.)

(Figures 4, 5, 6 and 7 are characteristic of type 6 strains.)

Fig. 8.—Edge of a giant proliferating colony showing an advancing edge of normal growth, and a following lysing area studied with resistant subcolonies. This colony measures 4 mm. in diameter. (× 100.)

Fig. 9.—Smear showing large faintly staining coccoid cells. (× 100.)

BACTERICIDAL PROPERTY OF WHOLE BLOOD OF WOMEN INFECTED WITH THE GONOCOCCUS

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The diagnosis of gonoccocal infection in women may be difficult especially when the disease is manifestly in the subacute or chronic state. The abundance of the vaginal microorganisms is always a handicap in the examination of smears and, in salpingitis, apparently but few gonococci appear at the cervix in the characteristic intracellular arrangement. Cultures are unreliable for the same reasons.

The observations of Torrey [1] and others that there are numerous immunologically distinct strains of gonococci have materially increased the efficiency of the complement fixation test by emphasizing the necessity for a polyvalent antigen. Its value has been estimated as greater than that of smears and cultures. According to Kolmer [2] the reaction is positive in 60% of the cases of pyosalpingitis. Agglutinins and precipitins have been demonstrated in the blood of infected persons but the percentage of reactions has been small, probably due to the fact that polyvalent antigens do not lend themselves to these reactions as readily as to the complement fixation test. In order to make a specific diagnosis of gonococcus infection in women, Torrey, Wilson and Buckell [3] recommend the use of the complement fixation test, smears, and cultures supplementing each other. Obviously it would be more advantageous to employ one test having a higher degree of accuracy.

In view of the results obtained with pneumococci and meningococci by Heist, S. Solis-Cohen and M. Solis-Cohen [4] using the Heist-Lacy reaction it would seem that the same reaction might apply to the gonococcus. This reaction is essentially a test of the bactericidal power of the whole blood upon the test organism. It is possible that with an organism of strong viability an exact numerical evaluation of the reaction might be obtained, but with the pneumococcus, meningococcus and gonococcus, so susceptible to adverse influences, only a rough estimation of bactericidal action can be made.

No reference can be found in the literature to the bactericidal property of the whole blood of an infected individual on the gonococcus. Scaltritti [5] has found that the whole blood of animals naturally resistant to the gonococcus, such as cattle, swine and sheep, possesses a bactericidal action on this organism. Whole blood from uninfected persons, he found, had no effect on the gonococcus.

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[1] J. Immunol., 1922, 7, p. 305.
[2] Infection, Immunity and Specific Therapy, 1917, p. 510.
[3] J. Infect. Dis., 1922, 31, p. 148.
[4] J. Immunol., 1918, 3, p. 261; 1922, 7, p. 1.
[5] Ann. de l'Inst. Pasteur, 1925, 39, p. 865.

Patients for whom a diagnosis of salpingitis had been made clinically and who were under treatment in the gynecological ward of the hospital furnished the specimens used in these experiments. These patients were chosen for several reasons. All were supposedly infected with the gonococcus. All were prospective subjects for salpingectomy, thus offering the opportunity for isolation of the infecting organisms from the uterine tubes. In case of surgical treatment the patient remained in the hospital long enough after operation so that their bloods could be tested with the homologous cultures. In all patients the infection was of sufficient duration to develop whatever immunity was possible. Control blood was taken from individuals who had probably never been infected.

The technic of the test was adapted to the peculiarities of the gonococcus.

The 18 hour growth on ascitic agar slants of each strain to be used was scraped off with a loop and a slightly turbid suspension in neutral broth was made. One fourth cc. of each suspension, was placed in as many agglutination tubes as there were bloods to be tested, including the control. All tubes containing suspensions were taken to the bedside where one cc. of each blood was added to one tube containing each suspension. The control blood was finally drawn and added to the suspensions so that the control organisms had been exposed to broth alone for the longest period of time.

All tubes were closed with rubber stoppers and incubated 24 hours. At the end of that time the clot was surrounded by a small amount of clear serum. There was no evidence of microbic growth nor could cocci be demonstrated microscopically in smears of the serum in most cases. A loop of about 2.5 mm. diameter was used to remove a drop of serum from each tube and spread it over the surface of an ascitic agar slant. These cultures were also closed with rubber stoppers and incubated 24 hours. The colonies developing were counted if they were discrete. If confluent, the growth was designated as infinite. Tubes showing no growth were called negative. Smears were made of all cultures to establish the identity of the organism.

The cultures of gonococci used in these experiments were isolated during the course of the work with the exception of that designated as A.M.S. (table 2) which was obtained from the Army Medical School. Strain 1 was isolated from an infected eye, strains 3 and 11 from joint fluid, 4 and 7 from the male urethra and 13 and 16 from the cervix. The other 10 strains were obtained from excised Fallopian tubes. An attempt was made to immunize rabbits with each strain and group the strains by agglutination tests but the reactions were not satisfactory.

A synopsis of the tests follows:

		Infection Not Proved	Infection Proved	Total
Blood specimens tested		43	15	58
Culture suspensions used		137	56	193
Culture suspensions showing bactericidal action	Number	47	24*	82
	%	34	43	42

* Exclusive of 11 homologous cultures.

It can be seen that the whole blood of individuals suspected of being infected with the gonococcus exerted a bactericidal action on 42% of the cultures used. The blood of those from whom the gonococcus was isolated showed the same percentage of reactions on cultures other than their own. It seems advisable, in a comparison of the results obtained with the infected group and that not proved infected, to exclude the results with the homologous strains, since, in a series of routine tests homologous strains would not be employed. The group including those in whom the infection was not proved was made up of 9 individuals

TABLE 1

EFFECT OF WHOLE BLOOD OF PATIENTS ON CULTURES OF THE INFECTING STRAINS OF GONOCOCCI

	Strains																
Blood	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
2	0	0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
3	+	+	0	..	..	..	..	..	..	..	..	..	..	..	..	..	..
5	..	..	..	(2)	0	..	..	..	..	..	..	..	..	..	..	..	..
9	..	..	..	..	0	+	+	0	(3)	..	..	..	..	..	..	..	..
8	..	..	..	..	+	+	+	(50)	(75)	..	..	..	..	..	..	..	..
11	..	..	..	..	0	..	..	..	+	(6)	0	..	..	..	..	..	..
12	..	..	..	..	..	..	..	..	..	..	..	(50)	+	..	..	..	..
14	..	..	..	..	..	..	..	..	..	..	..	+	+	0	(1)	..	..
15	..	..	..	..	..	..	..	..	..	..	..	(50)	(100)	0	0	..	..
16	..	..	..	..	..	..	..	..	..	..	..	..	+	0	+	(20)	+
17	..	..	..	..	..	..	..	..	..	..	..	..	+	+	+	+	(6)
Normal control	..	..	..	..	+	+	+	(14)	+	..	..	+	+	..	..	..	..

Uninhibited growth (+), sterilization (0), and partial inhibition (number of colonies) are indicated in all tables.

The tests with the homologous strains are underlined.

whose tubes were cultured and the gonococcus could not be found and 34 who were not operated. Since the percentage of reactions in this group was less than with the group proved infected, it is probable that some of them were not infected with the gonococcus and the pathologic condition was due to some other cause.

Table 1 shows the reactions obtained when the whole blood and the infecting strain were used, together with some heterologous strains. As a rule the homologous strain was completely sterilized. In a few cases the blood had no effect on any strain except the homologous one.

The viability of the gonococcus seemed to vary even when uniform conditions were maintained. Only a few colonies of a strain might grow from all the bloods including the normal control. An example of this variability is shown in the case of culture 8 in table 1. Occasionally unexplainable reactions occurred such as is shown in table 3 with the control blood and strain 14.

It is evidently impossible to make a diagnosis of gonococcus infection by this test unless a large number of strains is used or they are carefully selected as to their immunologic types. In table 2 patient 17 would be considered as noninfected when these three strains were used, yet a culture was obtained from her tubes after operation and it was acted

TABLE 2

Bactericidal Action of Blood on Several Strains of Gonococcus, with Special Reference to 15

Blood	Strain				
	A. M. S.	12	13	14	15
17	+	+	+	..	..
V	+	+	+	..	..
12	+	(50)	+	..	..
Control	+	+	+	..	..
16	+	+	+	+	(25)
18	+	+	+	0	+
15	(100)	(50)	(100)	0	0
14	+	+	+	0	(1)

upon by her blood as shown in table 3. On the other hand, the blood of patient 15 acted upon all of the strains used. All of the patients tested in table 3 were undoubtedly infected since all gave either a 3 + or 4 + complement fixation reaction using the same strains in an antigen. The bactericidal test would seem to indicate that two of these patients were not infected although their bloods gave a strong complement fixation reaction with the polyvalent antigen.

The bloods of 9 individuals, 8 males and 1 female, all undoubtedly never infected with the gonococcus have been used as control in 41 tests. Only 7 times did the blood of the control permit fewer surviving cocci than any of the test bloods. So the blood of uninfected individuals was an efficient control in 84% of the tests.

A consideration of the second and sixth cases in table 1 is interesting from the standpoint of vaccine therapy. Both cases were suffering from gonococcal arthritis, the gonococcus having been isolated from the synovial fluid in each. Each blood possessed a completely sterilizing activity upon the homologous strain. Treatment with an autogenous vaccine would seem to be of no value since the blood can already kill large numbers of the infecting organism. On the other hand treatment with heterologous strains would appear entirely futile.

Several interesting problems arise from a consideration of the results of these experiments. The whole blood of the infected person has a completely sterilizing power on the homologous organism yet the gonococci persist in the tissues for a long time. As a member of a group

TABLE 3

RESULTS OF BACTERICIDAL TESTS WITH BLOOD SPECIMENS GIVING POSITIVE COMPLEMENT FIXATION REACTIONS

Blood	Strain				
	13	14	15	16	17
17	+	+	+	+	(6)
16	+	0	+	(20)	+
A 1	+	+	+	+	+
A n	+	+	+	+	+
Control normal blood	+	(3)	+	+	+
Control broth	+	+	+	+	+

of organisms giving conspicuously nonspecific immune reactions the gonococcus in this test gives reactions approaching strain specificity. Is it possible that the bactericidal action is due, not to antibodies in the generally accepted sense, but to some other factor?

Although this test does not seem to offer any aid in the diagnosis of gonococcus infections yet the results in the small number of tests performed seem to indicate certain tendencies worthy of note.

CONCLUSIONS

The whole blood of normal persons possesses but little bactericidal action upon the gonococcus.

The blood of women having a gonococcal salpingitis exerts a strong bactericidal action on the gonococcus, most marked upon the infecting strain, indicating a tendency toward strain specificity.

This reaction is not dependable enough with heterologous strains to warrant its use as a diagnostic procedure.

Treatment of patients having chronic gonococcal infection with either stock or autogenous vaccines would seem theoretically to offer little hope of success.

TYPHOID VACCINATION BY MOUTH

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The work of several investigators indicates that certain bacteria introduced into the intestinal tract have antigenic action. Enlows [1] has reviewed the evidence and it need not be repeated here. Evidence of the antigenic action consists of positive agglutinin tests and increased resistance to infection. Evidence was obtained from both animal and human subjects. Results have not been uniformly positive but the majority of investigators report the demonstration of antibodies in the blood or increased resistance to infection following the oral administration of vaccines. The increased resistance in some cases was attributed to local rather than general immunity.

The experiments described in this paper were designed to determine; whether typhoid proteins were absorbed unaltered from the digestive tract as determined by the appearance of agglutinins; whether the agglutination titer would vary with the method of preparing the vaccine; whether "per os" vaccination is as effective as the subcutaneous method in stimulating the production of typhoid agglutinins; and to test the theory of increased cellular impermeability of the intestinal mucosa following the oral administration of typhoid vaccine.

EXPERIMENTAL WORK

Exper. 1.—To determine whether agglutinins appear in the blood of rabbits following the oral administration of typhoid vaccine.

Technic: A standard typhoid vaccine was prepared by washing off 24 hour extract agar slants of Bacterium typhosum with salt solution and sterilizing at 60 C. for 1 hour. The suspension was diluted to contain about 2 billion organisms per cc. The vaccine was administered through a catheter attached to a syringe. Two rabbits were used. One died after the fifth dose from causes other than the vaccine. The first two doses were 5 cc. volumes and the balance 10 cc. Three doses per week were given. The rabbit surviving, received a total of 140 cc. of vaccine. Food was kept before the rabbits at all times. No attempt was made to erode the intestinal mucosa by the use of bile. The agglutination titer was determined before the first dose of vaccine and at intervals during the experiment.

The results are given in table 1. The agglutination titer of the unvaccinated rabbits was 1:20. The titer of the surviving rabbit, nine days after receiving the

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[1] Public Health Report, U. S. P. H. S., 1925, 40, p. 639.

final dose, was complete agglutination 1:5000. The control was negative. The experiment demonstrates that agglutinins appear in the blood of rabbits following the oral administration of standard typhoid vaccine.

The increase in the agglutinating strength of the serum was observed. The last few doses appeared to have little effect on the titer and the administration of the vaccine by mouth was discontinued. The maximum immunity appeared about nine days after the last dose of vaccine.

TABLE 1

AGGLUTININS IN THE BLOOD OF RABBITS AFTER ORAL ADMINISTRATION OF TYPHOID VACCINE

Rabbit	Vaccine	Normal Titer	Doses Vaccine	Cc. Vaccine	Agglutination Titer
1	24 hour agar slant growth,	1:20	5	40	Died
2	1 hour at 60 C. (1)	1:20	19	140	1:5000

Exper. 2.—To determine whether the method of preparing the vaccine affects the production of agglutinins.

Technic: Vaccine 2 was prepared similarly to vaccine 1 except that the cultures were grown for 14 days. Vaccine 3 was prepared by culturing the organisms in Dunham's peptone water for 2 weeks and then heating to 60 C. for 1 hour. Vaccine 4 was prepared similarly to vaccine 1 except that it was heated to 98 C. for 1 hour. A suspension of live organisms, with unimportant exceptions, was used in testing for the presence of agglutinins. The use of live organisms is desirable in testing for immune reactions. In testing for the absorption of protein organisms subjected to the same treatment as the vaccine injected may be more desirable because of the possible modification of the bacterial protein.[2]

The results obtained are shown in table 2. Vaccine 2 produced results comparable to those obtained with vaccine 1. No agglutinins for live typhoid organisms appeared in the blood stream of rabbits receiving vaccines 3 and 4. That these rabbits were capable of producing agglutinins was demonstrated by 2 subcutaneous injections with the same vaccines. Unfortunately their ability to develop agglutinins following mouth vaccination with vaccine 1 was not determined. The results obtained in exper. 2, seem to indicate that the preparation of the vaccine may affect the development of specific typhoid agglutinins in rabbits following the oral administration of the vaccine. The typhoid protein may be sufficiently modified to check or prevent absorption through the mucosa. A second possible explanation is that some rabbits do not react to the oral administration of vaccines, due to an impervious intestinal mucosa. This explanation is tenable in view of the fact that the rabbits reacted to subcutaneous injections. Apparently the typhoid protein in these animals failed to reach the blood stream from the intestinal tract.

Exper. 3.—To determine whether vaccination by mouth is as effective as the subcutaneous method in stimulating the development of typhoid agglutinins in rabbits.

[2] J. Immunol. 1925, 10, p. 777.

Technic: Rabbits 2, 3, 4, 5, 6, 7 used in experiments 1 and 2 were given subcutaneous injections of the same vaccines used by mouth and the agglutination titer determined.

The results are given in table 3. It is evident from the results that subcutaneous injections are more effective than introduction by mouth in the stimulation of agglutinins: the strength of the agglutinins was increased in those rabbits which developed agglutinins as the result of vaccination by mouth and appeared in rabbits that had failed to produce agglutinins after oral administration. These findings can hardly be explained on the basis of daily variations in titer or as due to an increased titer sometimes associated with a second immunization.

TABLE 2

DETERMINATION OF EFFECT OF DIFFERENT PREPARATIONS OF VACCINE ON THE PRODUCTION OF SPECIFIC AGGLUTININS

Rabbit	Vaccine	Normal Titer	Doses Vaccine	Cc. Vaccine	Titer 9-14 Days after Last Dose
3	14 day agar slant growth,	1:10	16	140	1:5000
4	1 hour at 60 C. (2)	1:20	15	140	1:5000
5	2 weeks peptone water growth,	0	15	140	0
6	1 hour at 60 C. (3)	0	16	140	0
7	Vaccine 3 but 1 hour at 98 C.	0	11	100	0

TABLE 3

COMPARISONS OF AGGLUTININ PRODUCTION AFTER VACCINATION BY MOUTH, AND SUBCUTANEOUSLY

Rabbit	Vaccine	Normal Titer	Titer after Oral Vaccination	Subcutaneous Injections	Titer after Subcutaneous Injections
2	1	1:20	1:5000	4	1:8000
3	2	1:10	1:5000	4	1:20,000
4	2	1:20	1:5000	4	1:8000
5	3	0	0	2	1:40
6	3	0	0	0 Control	0
7	4	0	0	2	1:320

The fact that subcutaneous injections are more effective in stimulating the production of agglutinins does not indicate that they are more effective in stimulating the development of immunity to typhoid. A local tissue immunity appearing in the intestinal mucosa as the result of oral administration may be more effective than the general immunity resulting from subcutaneous injections. However, if the latter is proven to be true, any superiority of vaccination by mouth over subcutaneous vaccination against typhoid will be due mainly to simplicity of application rather than to the stimulation of greater immunity. This is true because subcutaneous vaccination stimulates the development of sufficient immunity.

DISCUSSION

Evidence in support of the theory of local tissue immunity and impermeability of the intestinal mucosa as a factor in typhoid immunity has been presented by Besredka. He believes that the apparently superior and more lasting immunity conferred by an attack of typhoid fever over that conferred by subcutaneous vaccination is due to the development in the intestinal mucosa of a local cellular immunity to the endotoxins and an impermeability to the organisms. He has shown that immune bodies appear in the blood following vaccination, by mouth, that these disappear although the animal remains immune and that they do not reappear following a second oral administration. He attributes the failure of immune bodies to appear in the blood following the second vaccination by mouth to an acquired immunity and impermeability of the intestinal mucosa.

Evidence concerning the acquisition of local tissue immunity and impermeability of the intestinal mucosa to typhoid following introduction by mouth can best be considered according to the following conceptions.

If immunity depends on the immunity and impermeability of the intestinal mucosa, susceptibility requires nonimmunity and permeability of the intestinal mucosa. In nonimmune animals immune bodies should appear in the blood following oral vaccination.

Vaccination by mouth should not stimulate production of immune bodies in the blood of nonimmune animals to the same extent as subcutaneous vaccination. With the development of intestinal mucosa immunity the amount of typhoid protein reaching the blood would decrease and finally cease. Immune bodies in the blood should disappear even though vaccination by mouth is continued. There should be no constant parallelism between immune bodies in the blood and immunity. An animal may be immune or develop greater immunity without having immune bodies apparent in the blood stream.

In animals immune as the result of an immunity and impermeability of the intestinal mucosa, immune bodies should not appear in the blood following oral vaccination. They should appear in such animals following subcutaneous vaccination.

Animals with an immune and impervious intestinal mucosa should be immune to the ingestion of live organisms. Such animals may not necessarily be immune to intravenous injection unless the progress of the disease depends on the infection of the intestinal mucosa.

Various protein substances should pass through the intestinal mucosa as readily as typhoid protein. These substances should pass through the intestinal mucosa after it has become immune and impermeable to typhoid protein. This is true because typhoid immunity following typhoid fever is largely specific.

The experiments described in this paper confirm the conclusions of Besredka that typhoid immune bodies appear in the blood of at least some animals following vaccination by mouth. They further demonstrate that subcutaneous vaccination will cause an increase in immune bodies in the blood after the animal ceases to react to oral vaccination and therefore that subcutaneous vaccination is more effective than oral vaccination in stimulating blood immunity. These facts suggest that impermeability of the intestinal mucosa may occur as the result of vaccination by mouth.

Our knowledge of the immunity conferred by mouth vaccination is not sufficiently complete for us to determine the comparative value of the method, when it should be used in preference to the subcutaneous method and when the two methods should be combined. In certain types of infection, such as infectious abortion in which the organisms gain entrance through both the oral and vaginal routes, immunity of the intestinal mucosa may not give complete protection. In certain diseases a general cellular and blood immunity as well as local tissue immunity is desirable. This may be true of some of the intestinal diseases. Since vaccination by mouth can not be depended upon to stimulate general blood immunity due to uncertainty of absorption on account of possible variation in the intestinal mucosa, it is questionable whether this method should be relied upon to the exclusion of the subcutaneous method. The choice of method should vary with the type of infection, the portal of entry of the organism and the nature of its toxic products. Erosion of the intestinal mucosa to insure greater exposure to the vaccine may enhance the value of vaccination by mouth but its practicability remains to be determined.

The absorption of unaltered or partially digested protein from the digestive tract has been well established experimentally. The route by which this protein gets into the circulation is not definitely known. The suggestion has been made that it may at times enter through the eroded or inflamed gastro-intestinal mucosa. Food allergy is accounted for in this way. It is possible that absorption may also take place when

the intestinal mucosa is intact.[3] Hektoen, Kanai, and Dragstedt [4] suggest that direct absorption may take place in the tributaries of the portal system.

CONCLUSIONS

Agglutinins appear in the blood of some rabbits following introduction of standard typhoid vaccine by mouth. Artificial erosion of the intestinal mucosa in such rabbits is unnecessary.

Subcutaneous vaccination is more effective than vaccination by mouth in stimulating the production of typhoid agglutinins in rabbits.

The comparative degree of immunity to typhoid conferred by the two methods of vaccination remains to be determined. The comparative rate at which immunity develops, considered separately from the appearance of agglutinins, is also unknown. Since subcutaneous vaccination affords adequate protection against typhoid any superiority of the oral method, considered from the practical standpoint, is limited to simplicity of administration and reduction in toxicity.

The absorption of typhoid protein from the intestinal tract of rabbits apparently decreases upon continued exposure of the mucosa to the protein.

[3] Kolmer, John A.: Infection, Immunity, Biologic Therapy, 1924, pp. 616, 668.
[4] J. Am. M. A., 1925, 84, p. 114.

THE GLUTATHIONE CONTENT OF ANIMAL TISSUES WITH REFERENCE TO TUBERCULOSIS

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In recent years, the subject of tissue oxidation in the animal body has attracted the attention of many investigators. Deviating from the old peroxide theory of Traube,[1] Wieland [2] postulated the theory that the initial cause of biological oxidation lies in the activation of hydrogen of the tissue substances rather than the activation of oxygen as formerly thought. From this standpoint, he interprets the reactions which are apparently true oxidations as dehydrogenation. Thunberg [3] has presented good evidence to support Wieland's theory by experiments with animal tissues. One of the most interesting findings in the study of tissue oxidation in recent years has been the isolation of a compound from animal tissues called "glutathione" by Hopkins.[4] This compound proved to be a dipeptide of cysteine and glutamic acid and it is claimed that it takes part in cellular oxidation and reduction in tissues as represented by the following scheme,[5] in which G — SH and G-S-S-G stand for the reduced and the oxided form of glutathione respectively, and M, the tissue substances that are oxidized, X, yet unidentified tissue constituent which acts as hydrogen donator.

$$\begin{matrix} G-SH \\ G-SH \end{matrix} \xrightarrow{O_2} \begin{matrix} G-S-H\text{—}O \\ G-S-H\text{—}O \end{matrix} \xrightarrow{M} \begin{matrix} G-SH \\ G-SH \end{matrix} + M\,O_2$$

$$X<^{H}_{H} + G-S-S-G \rightarrow X + 2G-S\,H$$

$$2\,G-SH + \tfrac{1}{2}\,O_2 \rightarrow G-S-S-G + H_2O$$

A more recent view advanced by Warburg [6] maintains that in biological oxidations molecular oxygen must first be activated by contact

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[1] Ber. d. deutsch. chem. Gesellsch., 1882, 15, pp. 659, 2421.
[2] Ibid., 1912, 45, p. 484; 1913, 46, p. 3327; 1914, 47, p. 2085.
[3] Skand. Arch. f. Physiol., 1920, 40, p. 1.
[4] Biochem. J., 1921, 15, p. 286.
[5] Hopkins, F. G., and Dixon, M.: J. Biol. Chem., 1922, 54, p. 527.
[6] Ber. d. deutsch. chem. Gesellsch., 1925, 58, B, p. 1001; Science, 1925, 61, p. 575. Warburg, O., and Sakuma: Pflüger's Arch., 1923, 200, p. 203.

or in combination with a catalyst which is usually a combination of an organic substance with a trace of iron. The trend of thought about tissue oxidation at present inclines more or less toward Warburg's view. The recent investigation by Harrison,[7] for example, has confirmed the view of Warburg in finding that a trace of iron markedly increased the oxidation of pure cysteine and reduced glutathione. This finding, however, does not minimize the importance of glutathione in the respiratory function of the cell; the experiments from Warburg's laboratory rather "show wherein it [SH-group] is significant. By combining with the SH group iron attains a reactivity which is greater than that of any of the known combinations." [8]

We are showing the relation of oxygen supply in animal tissues and the susceptibility of the organs to tuberculosis. The work of Novy and Soule [9] initiated and corroborated in this laboratory has shown that the respiration of tubercle bacilli plays an important role in their growth. As a result of elaborate animal studies,[10] it has been suggested that the slow development of tuberculosis in man is due to an insufficient oxygen supply in the animal body. It occurred to me that if glutathione is concerned in the respiration of the cell and that the development of tuberculosis in different tissues depend on the oxygen supply, it would be valuable to obtain quantitative estimations of glutathione in tissues and study its relation to the development of tuberculosis. From the biological standpoint, the quantitative estimation of glutathione alone would also give information of scientific value and interest.

METHOD.—For the quantitative determination of glutathione in tissues Tunnicliffe's method [11] seems to be the only one worthy of consideration. It consists in grinding the tissue with sand and extracting it three times with 10% trichloracetic acid. The resulting extract is then titrated with N/100 iodine solution using the nitroprusside reaction as an outside indicator. From the figures obtained by titration the amount of reduced glutathione is calculated according to the equation.

$$2\ R.\ SH + I_2 = R - S - S - H + 2\ H\ I$$

1 cc. N/100 I_2 = 2.5 mg. reduced glutathione

Three new modifications on the Tunnicliffe's method were utilized by me. The first was the use of a 1% starch solution as a direct indicator in the titration. This was preferred because the nitroprusside reaction as an outside indicator proved insufficiently sensitive to obtain a definite end point. The second change

[7] Biochem. J., 1924, 18, p. 1009.

[8] Warburg, O.: Science, 1925, 61, p. 575.

[9] J. Infect. Dis., 1925, 36, p. 60.

[10] Corper, H. J., and Goldberg, Max: Am. Rev. Tuberc., 1924, 8, p. 554; Corper, H. J., and Lurie, Max B.: J. Lab. and Clin. Med., 1925, 10, p. 95.

[11] Biochem. J., 1925, 19, p. 194.

was made in using 5% trichloracetic acid for the second and third extraction, and the third change was the use of the factor 2.34 mg. instead of 2.5 mg. the former figure being theoretically more correct than the latter. In order to note whether the modifications are satisfactory, 50 grams of rabbit's liver was extracted with trichloracetic acid and made up to a liter. From aliquot portions reduced glutathione was determined by the iodine titration, sulphate sulphur by Folin's method[12] and total sulphur by Benedict and Dennis' method[13] with the following results.

TABLE 1

GLUTATHIONE DETERMINATION BY DIFFERENT METHODS

	Cc. N/100 I_2 Solution	% Reduced Glutathione Calculated from Iodine Titration	Total Sulphur* as Mg. $BaSO_4$	Sulphate Sulphur as Mg. $BaSO_4$	% Reduced Glutathione Calculated from Organic Sulphur
100 cc. solution equivalent to 5 Gm. of liver	5.88	0.28	14.0	0.2	0.30
	5.99	0.28	13.5	0.4	0.29

* The figures represent the weight of $BaSO_4$ after the weight of $BaSO_4$ for blank determination for the reagents has been subtracted.

The perfect agreement between the percentage of reduced glutathione calculated from the iodine titration and the percentage calculated from organic sulphur indicates the accuracy of these modifications of the Tunnicliffe's method.

To determine reduced glutathione in tissues, animals were anesthetized with ether, and the blood was drained from the tissues by either cutting the jugular vein or by decapitation. A weighed amount of each tissue was ground and extracted with trichloracetic acid. It was finally titrated with the iodine solution. The extracts were usually tested by means of the nitroprusside reaction before titration with iodine and it was found that in every case there was a positive reaction in all the organs tested, except the serum in which there was no indication of the presence of glutathione either by the iodine titration or by the nitroprusside reaction. For the determination of glutathione in corpuscles oxalated blood was centrifuged and washed thoroughly with normal physiologic sodium chloride solution. The corpuscles were then laked by means of the addition of distilled water, the proteins precipitated with 10% trichloracetic acid, filtered and the determination was carried out in the above described manner. The figures reported in this work on corpuscles represent the percentage based upon the weight of blood corpuscle centrifugate in normal sodium chloride solution. The results of the quantitative estimation of reduced glutathione by iodine titration for normal animals are given in table 2. It has been found that the accuracy of the determination depends on the completeness of the drainage of blood from the tissues, the thoroughness of grinding the tissue, and the freshness of the sample.

In 1917 Burge and Neil[14] showed that the amount of catalase in animal tissue decreases with the progression of starvation. It is interesting to note whether glutathione decreases in the same manner as a result of starvation. For this purpose, animals were starved for a number of days and the quantitative estimation of glutathione was made with the results tabulated in table 3.

[12] J. Biol. Chem., 1905, 1, p. 151.
[13] Mathews: Physiological Chemistry, 1916, p. 981.
[14] Am. J. Physiol., 1917, 43, p. 58.

TABLE 2

PERCENTAGE OF REDUCED GLUTATHIONE IN TISSUES OF THE NORMAL ANIMAL

Animal	Sample	Liver	Lung	Muscle	Kidney	Spleen	Heart	Adrenal	Testicle	Ovaries	Brain	Lens	Pancreas	Marrow	Serum	Corpuscle
Rabbit, Female.....	A	0.33	0.13	0.037	0.14	0.24	0.07	0.20	....	0.12	0.10	0.35	....	0.09	0.00	0.07
	B	0.31	0.15	0.034	0.14	0.24	....	....	....	....	....	....	....	....	....	....
Female.....	A	0.34	0.15	0.040	0.16	0.24	0.08	0.11	....	0.22	0.08	0.31	....	0.07	0.00	0.05
	B	0.33	0.13	0.041	0.16	....	....	....	....	....	....	....	....	....	....	....
Rat, Male.....	A	0.23	0.12	0.034	0.16	0.15	0.07	....	0.10	....	0.11	....	....	....	0.00	0.04
	B	....	....	0.032	0.15	....	....	....	....	....	....	....	....	....	....	....
Male.....	A	0.23	0.10	0.036	0.20	0.17	0.08	....	0.20	....	0.12	....	....	....	0.00	0.06
	B	....	....	0.036	....	....	....	....	....	....	....	....	....	....	....	....
Dog, Female.....	A	0.23	0.10	0.050	0.15	0.09	0.07	....	...	....	0.08	0.37	0.12	....	....	....
	B	0.23	0.10	0.046	0.15	....	0.07	....	....	....	0.07	....	0.19	....	....	....
Male.....	A	0.18	0.08	0.040	0.15	0.09	0.09	....	0.07	....	0.05	0.22	0.08	....	0.00	....
	B	0.19	0.08	0.040	0.12	0.09	0.10	....	0.04	....	0.06	....	0.09	....	....	....
Male.....	A	0.20	0.06	0.061	0.14	0.08	0.08	....	0.06	....	0.05	0.20	....	0.013	....	0.02
	B	0.20	0.06	0.057	0.14	0.08	0.08	....	0.06	....	0.05	....	....	....	....	0.02
Guinea-pig, Male.....	A	0.32	0.12	0.033	0.20	0.21	0.07	....	0.13	....	0.08	0.23	....	....	0.00	0.06
	B	0.32	....	0.033	0.19	....	....	....	....	....	....	....	....	....	....	....
Male.....	A	0.26	0.13	0.040	0.19	0.23	0.07	0.13	0.15	....	0.11	0.43	....	....	0.00	0.12
	B	0.25	....	0.040	0.19	....	....	....	....	....	....	....	....	....	....	....
Average for rabbit.....		0.33	0.14	0.038	0.15	0.24	0.08	0.16	....	0.17	0.09	0.33	....	0.08	0.00	0.06
Average for rat.....		0.23	0.11	0.033	0.18	0.16	0.08	....	0.15	....	0.12	....	....	....	0.00	0.05
Average for dog.....		0.21	0.08	0.050	0.14	0.09	0.08	....	0.06	....	0.06	0.27	....	....	0.00	0.02
Average for guinea-pig.....		0.29	0.13	0.037	0.19	0.22	0.07	0.13	0.14	....	0.10	0.33	....	....	0.00	0.09
Average for the 4 animal species.....		0.27	0.12	0.040	0.17	0.18	0.08	0.15	0.12	....	0.09	0.31	....	0.08	0.00	0.06

GLUTATHIONE CONTENT OF THE TISSUES OF NORMAL ANIMALS

It is seen from the average value obtained for the four animals presented in table 2 that the order of the organs arranged according to their glutathione content is as follows: lens, liver, spleen, kidney, adrenals, testicles and lung, brain, bone marrow and heart, and muscle. With the exception of the lens, this order represents fairly well the order of chemical activity ascribed to each organ. Salkowski and Yamagiwa [15] found certain animal organs to stand in the following order in their oxidizing power toward salicylic aldehyde: liver, 100.0; spleen, 80.0; kidney, 15.5; pancreas, 2.0; muscle, 1.0. It is remarkable that this order which represents the oxidizing power of the tissues is in exact agreement with the one arranged according to the glutathione content of the animal tissues as enumerated by the writer. This finding is another evidence showing that the glutathione is essentially concerned in the oxidative processes in the tissues.

The reason for lens containing more glutathione than other tissues remains unexplained. One probable explanation for it seems to be that the dipeptide has special optical properties suited to the part played by the lens.

It is specially to be noted that the glutathione content of a certain organ for each animal species is very constant and appears to be characteristic for this animal. For example the highest glutathione value for liver is found in rabbits with 0.33% as an average, and the lowest value is found in dogs with an average of 0.21% while guinea-pigs and rats occupy an intermediate position. In the case of spleen, the rabbit contains as much as 0.24% while dogs contain only 0.09% as an average. The values for heart are very constant throughout the four species tested. It is also to be noted that the glutathione content of testicles runs in the order of the fertility of the species; namely, rats, which are the most fertile of the four tested, have the highest average of 0.15% followed consecutively by guinea-pigs with 0.14% rabbits with 0.10% (table 3) and dogs with 0.06%. The latter being the least fertile of the four.

Blood which has a low oxidizing power itself possesses very little glutathione. Tunnicliffe [11] has reported the complete absence of glutathione in the whole blood while Arnold [16] reported the presence of a

[15] Central. f. deutsch. med. Wissensch., 1894, 32, p. 913. Abelous and Biarnes (Arch. de Physiol., 1895, 1, p. 195) arrange the tissues in a slightly different order for their oxidizing power toward salicylic aldehyde.

[16] Zeitschr. f. physiol. Chem., 1911, 70, pp. 300, 314.

TABLE 3

PERCENTAGE OF REDUCED GLUTATHIONE IN THE TISSUES OF FASTING ANIMALS

Animal	Fasting Period	Samples	Liver	Lung	Muscle	Kidney	Spleen	Heart	Adrenal	Testicle	Brain	Lens	Marrow	Serum	Corpuscle
Rabbit	24 hours	A	0.32	0.16	0.040	0.13	0.23	0.09	0.12	0.11	0.07	0.32	0.07	0.00	0.06
		B	0.31	0.17	0.039	0.12	....	....	....	....	....	....	....	....	....
	48 hours	A	0.28	0.15	0.056	0.10	0.23	0.09	0.22	0.10	0.07	0.24	0.09	0.00	....
		B	0.29	0.14	0.060	0.10	....	....	....	0.10	....	....	....	....	....
	72 hours	A	0.30	0.18	0.056	0.11	0.18	0.08	....	0.09	0.08	0.31	0.08	....	....
		B	0.30	0.17	0.056	0.11	....	0.07	....	....	....	....	....	....	....
Rat	3 days	A	0.27	0.07	0.019	0.09	0.12	0.08	....	....	0.07	....	....	....	....
		B	....	....	....	....	....	....	....	....	....	....	....	....	....
	3 days	A	0.28	0.07	0.017	0.12	0.11	0.04	....	....	0.08	....	....	....	....
		B	....	....	....	....	....	....	....	....	....	....	....	....	....
	3 days	A	0.22	0.07	0.025	0.09	0.11	0.06	....	....	0.08	....	....	....	....
		B	....	....	....	....	....	....	....	....	....	....	....	....	....
	5 days	A	0.21	0.09	0.023	0.17	....	0.05	....	....	0.09	....	....	0.00	0.06
		B	....	....	0.021	0.17	....	....	....	....	....	....	....	....	....
Dog	3 days	A	0.18	0.04	0.047	0.14	0.10	0.09	....	0.08	0.06	0.27	....	0.00	0.02
		B	0.16	0.04	0.042	0.14	0.10	0.09	....	0.04	0.07	....	....	....	0.01
Guinea-pig	2 days	A	0.22	0.12	0.041	0.17	0.23	0.06	....	0.17	0.08	0.32	....	0.00	0.09
		B	0.24	....	0.042	0.16	....	....	....	....	....	....	....	....	....
	2 days	A	0.27	0.11	0.039	0.21	0.22	0.09	....	0.17	0.08	0.39	....	0.00	0.08
		B	0.27	....	0.041	0.21	....	....	....	....	....	....	....	....	....
	7 days	A	0.20	0.12	0.048	0.17	0.22	0.07	0.16	0.17	0.08	0.23	....	0.00	0.10
		B	0.20	....	0.047	....	....	....	....	....	....	....	....	....	....
Mean value for rabbit			0.30	0.16	0.050	0.11	0.21	0.08	0.17	0.10	0.08	0.29	0.08	0.00	0.06
Mean value for rat			0.24	0.08	0.021	0.12	0.11	0.06	....	....	0.08	....	....	0.00	0.06
Mean value for dog			0.17	0.04	0.045	0.14	0.10	0.09	....	0.06	0.06	0.27	....	....	0.02
Mean value for guinea-pig			0.23	0.12	0.043	0.18	0.22	0.07	0.16	0.17	0.08	0.31	....	....	0.09

substance in blood giving the nitroprusside reaction. In order to learn the cause for the discrepancy between the observations of these two investigators the serum and the corpuscles were separated, and after laking the latter the dipeptide content was determined in each fraction. In all the animals tested a small amount of glutathione was found in the corpuscle but it was completely absent from the serum. These results are corroborated by the work of Holden.[17] Therefore the failure of Tunnicliffe to find glutathione in blood was probably due either to the unreliability of the nitroprusside reaction, or to the failure to lake the blood corpuscle before extraction with trichloracetic acid.

GLUTATHIONE CONTENT AND ORGAN SUSCEPTIBILITY TO TUBERCULOSIS

The greater susceptibility of the lung over other organs to tuberculosis can be explained as due to oxygen supply since the lungs are the portals of oxygen supply to the body tissues, and the lung tissue which lies between the blood capillaries and the alveolar spaces must have an oxygen tension more or less equal to that of the capillary blood or of the alveolar spaces.

When we come to consider the difference, however, in the rate of tuberculous involvement among the organs of the same animal and among the same organs of different species (Corper-Lurie[10]), from the standpoint of cellular oxygen supply as influenced by the glutathione content there does not seem to exist any relation between the glutathione content and the rate of development of tuberculosis in tissues. Take the case of the rabbit, for instance, the liver is very resistant and the spleen and the kidney are moderately susceptible to tuberculosis while the dipeptide content is in the reverse order. The glutathione content of the lung of the rabbit, guinea-pig and dogs is in the order of susceptibility to tuberculosis, but that of the liver is in the reverse order of susceptibility. The spleen of the guinea-pig is most susceptible of the three species yet the glutathione content occupies an intermediate position between the rabbit and the dog. Regarding the view of organ susceptibility being influenced by oxygen supply it does not seem possible at present to draw any definite conclusions since the oxygen supply of the cell may be influenced by other factors such as that exerted by thyroxin, oxidases and by the acid-base equilibrium of blood. The results of this investigation on glutathione seem to indicate that there is no relation between the glutathione content of the tissues and the rate of the development of tuberculosis in the different tissues.

[17] Biochem. J., 1925, 19, p. 727.

GLUTATHIONE CONTENT IN FASTING ANIMALS

The glutathione content of the various organs of rats, guinea-pigs, rabbits and dogs, as seen from the figures in table 3, unlike the catalase content, remains constant during fasting. There have been only two exceptions to this; one the glutathione content of the muscle in rats which decreased from 0.033% for the normal animal to 0.021% for the fasting, and the other was the lung of the dog whose normal figure of 0.08% decreased to 0.04% during starvation. Before any explanation for this can be given more data must be obtained to note whether this decrease is a constant finding and whether it is proportional to the length of time of fasting. A possible explanation for the constancy of the glutathione content during fasting and the decrease of catalase content seems to reside in the fact that catalase is an unstable substance whose content can be decreased or increased according to the metabolic conditions of the animal body and that its constancy is not essential for the respiration of the cell, while on the other hand, glutathione is a stable cellular component whose main function lies in cellular respiration and its constancy is essential to the life of the cell. Catalase and glutathione differ essentially also in the thermoinstability of the former and the thermostability of the latter.[5]

SUMMARY AND CONCLUSIONS

The glutathione content of the various organs in some of the common experimental animals, rat, guinea-pig, rabbit and dog, reveals a constancy in the amount in each organ of the individual species and a characteristic constant amount for each of the different species. The glutathione occurs in the order of decreasing amounts in the lens, liver, spleen, kidney, adrenals, testicles, lung, brain, marrow, heart, and muscle. This order is in exact agreement with that given by Salkowski and Yamagiwa for the oxidizing power of certain animal tissues toward salicylaldehyde. Blood serum contains no glutathione while the corpuscles reveal a small determinable amount.

No relation exists of the species tested to tuberculosis, between the glutathione content of the various organ tissues and the organ susceptibility.

The glutathione content of animal organs remains constant during fasting, probably because of the stability of this compound in the animal body as well as in vitro in distinction to the instability and variability of catalase.

INVESTIGATIONS ON ROCKY MOUNTAIN SPOTTED FEVER IN COLORADO

ONE PLATE

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Although the pioneer work on Rocky Mountain spotted fever was done in Idaho, interest soon became centered around the disease as it exists in Montana, particularly in the Bitter Root Valley, and most of the investigations have been conducted in this region or with material collected there. The infection, however, is quite generally distributed throughout the Rocky Mountain states, and it would seem that independent study in different sections of this area would be valuable and might lead to the solution of some problems as yet unsolved. A few important publications have appeared from states other than Montana, but in Colorado very little study has been made of the disease, although a number of cases occur here annually. Campbell [1] in 1916 published a paper which dealt mainly with the life cycle of the transmitting tick. Since then, to my knowledge, nothing has appeared from this state except an occasional case report.

In view of these facts the studies here presented were undertaken. The investigation was divided into the following parts: an analysis of the official statistics, experimental study of patients diagnosed to have Rocky Mountain spotted fever, and attempts to produce the disease experimentally from ticks collected in Colorado.

Before approaching the problem as outlined, it seemed advisable to obtain a strain of the Bitter Root Valley infection for first hand observation. Through the kindness of Dr. R. R. Parker two infected guinea-pigs were received Jan. 19, 1923, from the U. S. Government laboratories in Montana, and this strain was kept alive for three months. The clinical and postmortem findings were in accordance with the accepted description of the disease in guinea-pigs. Figure 1 in the accompanying illustration is given for comparison with the other figures. It shows a part of a section through the necrotic penis of guinea-pig 6 of this series. In the field are large numbers of the double lanceolate form of the

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[1] Colorado Med., 1916, 13, p. 209.

causative organism (Dermacentroxenus rickettsi), mainly in the cytoplasm of endothelial cells. The organisms are about one mikron long and from .2 to .3 of a mikron wide. They are stained a delicate reddish violet and are somewhat indefinite in outline. Some of these forms seem to be surrounded by a light pinkish halo.

Zenker fixation and Giemsa staining technic with thin tissue sections as described by Wolbach [2] were used for this and all other preparations. The drawings were made with the camera lucida and the outlines were slightly accentuated for purposes of reproduction.

INCIDENCE STATISTICS FOR COLORADO

Statistics on Rocky Mountain spotted fever for Colorado were available for the years 1918-1924 inclusive. A total of 19 cases with 10 deaths were reported as follows:—1918, one death in Denver; 1919, two deaths, one in Fremont Co. and one in Larimer Co.; 1920, one death in Garfield Co.; 1921, two deaths, one in Saguache Co. and one in Moffat Co.; 1922, four cases, one death in Chaffee Co. and a recovery in each of the following counties, Moffat, Garfield, and Boulder; 1923, three cases, one death in Denver (patient came from Wyoming), one death and one recovery in Moffat Co.; 1924, six cases, one death in Moffat Co. and the following recoveries: one in Rio Blanco Co., one in Moffat Co., one in Garfield Co., and two in Larimer Co.

That these statistics are not complete may be concluded from the following facts. Four cases came under my observation during the springs of 1922 and 1923 which were not reported. In one of these the Rocky Mountain spotted fever organism was found. In response to a questionary, seven additional cases were reported. On the other hand, one must not overlook the fact that cases reported may not have acquired the infection in this state.

STUDY OF FIVE CASES IN COLORADO

Case 1.—April 2, 1922, an adult woman in Boulder had a moderate fever and other symptoms of malaise; a tick was found attached and engorged; no rash developed. The patient recovered.

An attempt to infect a guinea-pig with this tick failed. The Rocky Mountain spotted fever organism was not found in sections of this tick.

Case 2.—April 6, 1922, a boy in Boulder had a high fever and complained of generalized aching; no purpuric rash developed. The patient recovered in one week. This patient had been in the mountains on April 2. Two ticks were found attached that evening, and the boy had fever. This boy had also been in the mountains a week before this date. There was no record of a tick bite from this earlier trip. However, the boy had felt a lump in the axilla and had removed it without investigating its nature. This may have been a tick.

[2] J. Med. Res., 1919, 41, p. 1.

Ten cc. of citrated blood from this patient were injected intraperitoneally into a male guinea-pig April 6, but the pig did not develop any signs or symptoms of disease.

Case 3.—April 8, a boy, aged 10, was thought to have Rocky Mountain spotted fever on the basis of the clinical picture. He was acutely ill with a high fever, delirium, and severe general aching and tenderness; a macular hemorrhagic rash appeared; later large purpuric plaques developed generally over the body, most severe on the upper back, chest and on the shoulders. These areas became necrotic and after two months cleared up. The condition was complicated by double otitis media. There was severe pain in the abdomen, and for several days movements were involuntary and contained much mucus. The patient left the hospital after nearly two months.

April 14 a guinea-pig was inoculated with 5 cc. of blood from this patient. Moderate fever developed. April 24 the animal was killed. The testes were markedly congested. The spleen was enlarged. No spotted fever organisms were found.

April 26 two more guinea-pigs were inoculated with 5 cc. of citrated blood from this patient. The following is the record of guinea-pig 7:—April 26, temperature before inoculation, 100; April 27, 102; April 28, 102.5; April 29, 101.7; April 30, 101.7; May 1, 102.8; May 2, 101.8; May 3, 102.9 (marked swelling of the scrotum); May 4, 102.2; May 5, 102; May 6, 102.9; May 7, 102.8; May 8, 102.9; May 9, 101.9 (profuse diarrhea); May 10, 100. (bleeding from the rectum). The animal was killed. There were hemorrhagic areas in the region of the scrotum, the peritoneum was markedly inflamed and thickened, the spleen presented a sago appearance, the bowels were distended with fluid. Organisms indistinguishable from the Rocky Mountain spotted fever organism were found in sections of the peritoneum and spleen.

A subinoculation was made from this guinea-pig with similar results.

There was no history of a tick bite obtained in this case. The boy had run away from his home in Longmont, and had been in Wyoming for two days when he became sick. The findings would confirm the diagnosis of Rocky Mountain spotted fever even in the absence of a history of tick bite, which was perhaps overlooked. The infection was probably not acquired in Wyoming, as the boy was sick when he returned from his two day stay, and this would not allow time for the normal incubation period of three to four days after a tick bite.

Case 4.—In May, 1922, two cases were reported from Salida. A man, aged 64, died from the infection complicated by bronchopneumonia before inoculations could be made. The other, aged 24, recovered. He had been in the mountains and had removed a tick from the right hypochondrium several days before he became ill. He had a high temperature and a general purpuric rash which covered even the soles of his feet. When seen May 5 for the purpose of making inoculations, he was convalescing. No history of diarrhea or bloody stools could be obtained.

Four guinea-pigs were inoculated May 5 from this patient. All four animals developed moderate fever, up to 103, the coat of fur became rough, and the scrotum and paws became swollen and red. One developed a slough in the region of the scrotum which was very sluggish in healing. All four animals developed anorexia and diarrhea with bloody stools. One died June 3, two died June 4, and the last one was killed June 12. Postmortem examination revealed findings typical of Rocky Mountain spotted fever, and in addition a marked inflammatory thickening of the peritoneum and diffuse hemorrhagic areas in the mucosa of the gastro-intestinal tract. Organisms similar to the Rocky Mountain spotted fever organism were found in nearly all sections studied. The intestinal tract was not examined microscopically.

Case 5.—In the early spring of 1923 an adult male of about sixty, in Boulder, developed high fever and generalized pain and tenderness several days after a tick bite. No skin rash appeared. The patient recovered.

A guinea-pig was inoculated with 5 cc. of blood from this patient, but it did not develop any symptoms or signs of disease.

EXPERIMENTS WITH WILD TICKS

The following attempts were made to produce the disease experimentally in guinea-pigs with wild ticks collected in the vicinity of Boulder.

Exper. 1.—Guinea-pig 1: Three wild ticks were placed on this animal April 1, 1922. All three ticks became attached within 24 hours. Temperature record:—April 2, 101; April 3, 102.8; April 4, 99; April 5 (?); April 6, 100.7; April 7, 100.7; April 8, 102.6; April 9, 102.3; April 10, 103.3; April 11, 102.8; April 12, 102.3.

April 12 guinea-pigs 4 and 5 were inoculated from guinea-pig 1 which was then killed. Postmortem showed marked emaciation, roughness of fur, edema, and hemorrhagic areas at the site of tick bites. No other pronounced gross lesions were noted, but organisms indistinguishable from D. rickettsi were found in sections of the skin, spleen, and testes.

Two of the ticks were prepared for microscopic study. The third tick had not engorged and was discarded. In one of these ticks rickettsia-like bodies were found in large numbers. Figures 2 and 3 show a section of the oviduct in which are seen paired double lanceolate forms with a staining reaction and morphology characteristic of rickettsia bodies.

A large spirochete was found in sections of the oviduct near the ovary (figs. 2 and 3). The turns of the spirochete are short and numerous. There seems to be a definite sheath or membrane around the organism. It is stained purple and is granulated. In many fields the organisms seemed to be breaking up into granules of different sizes and shapes (fig. 3). The spirochete was not found in the tissues of the guinea-pig infected by this tick, and the blood was not examined.

Guinea-pigs 5 and 6, inoculated from guinea-pig 1 on April 12, presented moderate temperature curves, very much like guinea-pig 1. These animals were observed for four weeks when both had marked diarrhea and bleeding from the rectum.

April 21, guinea-pigs 15 and 16 were inoculated from guinea-pigs 4 and 5, respectively, and guinea-pigs 4 and 5 were then killed.

Figure 4 shows a section through a hemorrhagic area in the intestinal wall of guinea-pig 5. Here large numbers of rickettsia-like bodies are seen in the cells of the mucosa and submucosa.

Guinea-pigs 15 and 16 had temperature curves similar to that of guinea-pig 1. The animals were kept alive for 17 days, when marked emaciation, diarrhea, bleeding from the rectum, and lymph gland enlargement were noted. No subinoculations were made.

Guinea-pig 16 only was studied microscopically. Figure 5 shows a section of a small artery in the submucosa of the stomach wall which grossly showed diffuse hemorrhagic lesions. Here may be seen large numbers of rickettsia bodies in endothelial cells and muscle cells. There is a proliferation of endothelial cells which in some fields resulted in total occlusion of small vessels. A large number of filamentous, rod, and coccus-shaped microorganisms were found in this section, some of which are shown in fig. 6, where also some double lanceolate rickettsia bodies are noted.

Exper. 2.—April 12, 1922, four wild ticks collected near Boulder were placed on guinea-pig 10. Temperature record: April 29, 100.2; April 30, 100.9 (all four ticks attached); May 1, 100.5; May 2, 100.5; May 3, 100.5; May 4, 100.5; May 5, 101; May 6, 101 (cage off and all ticks lost); May 7, 102.1; May 8, 102.1; May 9, 102.1; May 10, 102.7 (scrotum markedly swollen); May 11, 102.7 (bleeding from rectum and paws swollen); May 12, 103; May 13, 102.2; May 14, 101.7; May 15, 95.6; May 16, animal found dead.

Attention is called to the fact that bleeding from the rectum was noted in this guinea-pig on the twelfth day after the ticks became attached.

Autopsy showed hemorrhagic areas in the region of the tick bites, paws, scrotum, and stomach; liver was mottled, spleen had a granular appearance and was dark red and enlarged.

In spite of the very characteristic picture presented in this guinea-pig, the Rocky Mountain spotted fever organism was not found. Unfortunately the tissues from the hemorrhagic stomach wall were lost.

Exper. 3.—June 19, 1922, six wild ticks were placed on guinea-pig 17 in a wire capsule. Two became attached. The animal was observed until July 15, and did not develop any symptoms or signs of disease. There was some slight elevation of temperature noted in this guinea-pig, but this was very likely due to the influence of very hot weather as the control pigs kept under similar conditions showed a similar elevation.

Exper. 4.—June 19, 1922, three wild ticks were placed on guinea-pig 19 in a wire capsule. Two became attached, one fully engorged. The animal was observed until July 15 and did not develop any symptoms or signs of disease. The ticks were sectioned and rickettsia-like organisms were not found.

DISCUSSION

According to official statistics, Rocky Mountain spotted fever exists in Colorado with an average annual incidence of 2.57 cases and a mortality of 1.5 cases. That these statistics are inadequate has already been pointed out. The mortality figures are perhaps nearly correct but the actual morbidity is higher. The mortality rate lies between that of 5% in Idaho and 87% in the Bitter Root Valley in Montana. There is no reason to question the diagnosis in these 18 cases reported. One of them was studied in detail by the author and the diagnosis was confirmed. Although the transmitting tick (Dermacentor andersoni) is abundantly present in all the mountainous regions of the state, cases are reported from only a few counties. We must therefore assume that there are certain centers of infection in Colorado.

An organism indistinguishable morphologically from D. rickettsi was found in two of the six clinical cases that were studied, cases 3 and 4. In both of these cases the disease in patients and in guinea-pigs was fairly typical of Rocky Mountain spotted fever. It differed, however, in manifestations of gastro-intestinal symptoms and lesions. In case 3 pain in the abdomen, distention and diarrhea were complaints. It

was impossible to obtain a complete clinical history of case 4, symptoms referable to the gastro-intestinal tract were not recorded. However, guinea-pigs inoculated with blood from these patients developed profuse diarrhea and bleeding from the rectum, and diffuse hemorrhagic areas were found in the stomach and intestines.

The attempt to produce the disease experimentally by the bite of ticks collected in the mountains seems to have been successful in exper. 1. An organism indistinguishable morphologically from D. rickettsi was found. The infection thus produced was not very severe. It differed from the typical disease in guinea-pigs by a lower temperature curve, by diarrhea and bleeding from the rectum, and by the production of hemorrhagic lesions in the stomach and intestines.

These experimental studies seem to justify the conclusion that Rocky Mountain spotted fever exists in Colorado; however, the atypical gastro-intestinal symptoms in many of these cases demand some consideration. To my knowledge similar observations have not been recorded. Wolbach [2] gives constipation and occasionally moderate vomiting as a part of the clinical picture, but nowhere does he mention diarrhea or intestinal hemorrhage. In his description of the pathology of the disease in animals, he says: "The peritoneal surfaces and intestinal tract are normal." In his summary on the pathologic changes in man, he mentions several small thrombi in the stomach wall of two of his cases, and the finding of a few micro-organisms in one case. Le Count [3] does not mention any lesions in the stomach or intestines.

These atypical symptoms did not attract serious attention at first. They were thought to be due to intercurrent infection, and not a part of the clinical manifestations of Rocky Mountain spotted fever. The finding of rickettsia-like bodies in sections of the stomach and intestines of guinea-pigs with these lesions seems to indicate that the gastro-intestinal involvement is a part of Rocky Mountain spotted fever. The gastro-intestinal tract of a normal, noninfected guinea-pig was studied microscopically. Rickettsia-like bodies were not found. A spore-like organism was present in large numbers (fig. 7). Its size, staining reaction, and morphology differentiates it readily from D. rickettsi.

It seems therefore that a causal relationship must exist between certain cases of Rocky Mountain spotted fever in Colorado and the atypical symptoms and lesions under discussion.

[3] J. Infect. Dis., 1911, 8, p. 421.

These observations raise a number of questions. Is the localization of the organism in the gastro-intestinal tract merely a late manifestation of a very mild form of the infection? Has the organism in question acquired new pathogenic properties in this locality, producing these atypical symptoms and lesions? Are the rickettsia bodies here superimposed upon a coincident infection which has weakened the gastro-intestinal tract and thus made it more susceptible?

Whether or not the other micro-organisms found in the stomach wall (fig. 6) are pathogenic could not be determined. This is also true of the spirochete shown in figs. 2 and 3. The possibility that other diseases than Rocky Mountain spotted fever can be produced by the wood tick (D. andersoni) must not be overlooked. Many cases of paralysis following tick bites have been reported by McCornack [4] and Bassoe [5] and others. The organism responsible for this paralysis remains to be discovered.

In Colorado, more or less marked general symptoms of indisposition often develop after tick bite. These are not at all characteristic of Rocky Mountain spotted fever, but there seems to be such a definite connection with tick bite that it appears that the tick must also here be considered in an etiological role.

Three of the five clinical cases reviewed in this paper (1, 2 and 5) are examples of such an infection. These patients all had a history of tick bite, moderate fever, and general symptoms of malaise, but they did not develop the skin lesions which characterize Rocky Mountain spotted fever, and which are as a rule even more marked in the mild than in the severe type of the disease. The condition just described is so common that physicians sometimes speak of it as "tick fever," a definite entity, in contradistinction to Rocky Mountain spotted fever.

Such a distinction is perhaps not justified in the light of our present knowledge because the number of cases accurately studied is far too small. No symptoms or lesions characteristic of Rocky Mountain spotted fever were seen in guinea-pigs inoculated with blood from these patients, and no organisms resembling D. rickettsi were found.

These findings seem to support the view that this type of infection is not Rocky Mountain spotted fever. However, the presence of rickettsiae may have been overlooked and this may be simply another demonstration of the well established conception of difference in virulence of virus or

[4] J. Am. M. A., 1921, 77, p. 260.
[5] Arch. Neurol. & Psychiat., 1924, 12, p. 564.

degree of resistance in the individual. If this clinical syndrome is not Rocky Mountain spotted fever and if it is really due to tick bite, further work may show its true nature and a causative organism other than D. rickettsi may eventually be demonstrated.

CONCLUSIONS

Rocky Mountain spotted fever exists in Colorado with an average annual incidence of more than 2.57 cases and a mortality of 1.5 cases. The mortality rate lies between that of Idaho and the Bitter Root Valley in Montana.

The disease is widely spread throughout the mountainous regions of the state with certain centers more heavily infected than others.

The disease in several patients and in guinea-pigs infected from these patients and from wood ticks differed from the typical Montana form in the exhibition of marked gastro-intestinal symptoms and lesions.

Many micro-organisms, other than D. rickettsi, are found in local ticks. That these have pathogenic properties has not been demonstrated, but further work seems indicated.

Certain symptoms of malaise, not typical of Rocky Mountain spotted fever, frequently follow tick bites in this state. Evidence supports the view that they are due to the tick bite, but are not a part of the clinical picture of Rocky Mountain spotted fever.

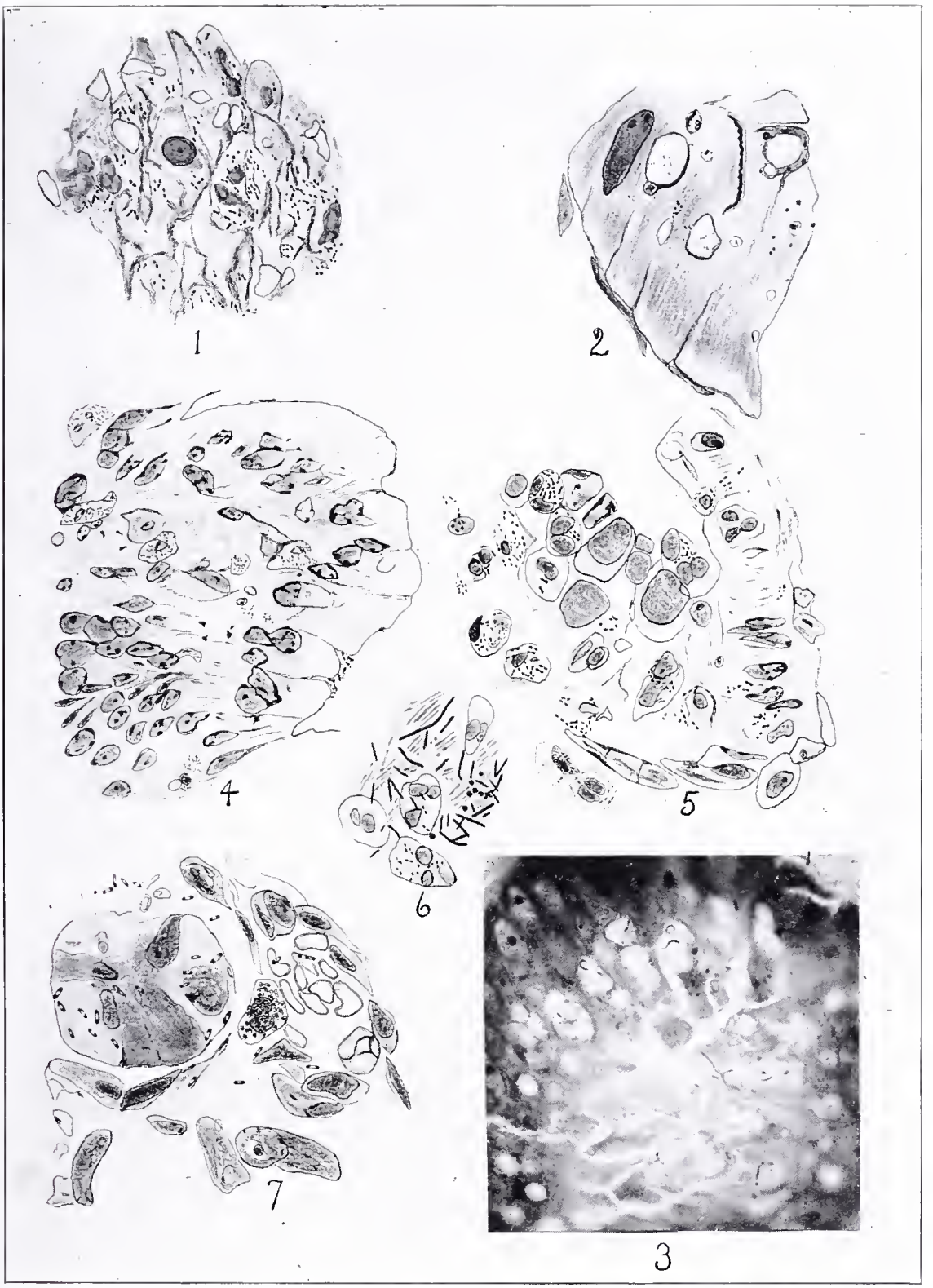

Fig. 1.—Section of necrotic penis of guinea-pig 6 (Montana strain), showing many double-lanceolate forms of the parasite. × 1130.

Fig. 2.—Section of oviduct of tick, showing double-lanceolate form of rickettsia, large spirochete, cocci and ring shaped bodies. × 500.

Fig. 3.—Section of oviduct of tick, as in fig. 2. Shows many spirochetes. Rickettsiae not seen because of low magnification. × 375. Photomicrograph.

Fig. .—Section of intestine of guinea-pig 5. Shows rickettsiae in endothelial cells. × 600.

Fig. 5.—Arteriole in submucosa of stomach wall of guinea-pig 16. Shows rickettsiae in endothelial and muscle cells. × 1130.

Fig. 6.—Small field from stomach wall of guinea-pig 16. Shows rickettsiae, cocci, rods and filaments. × 1130.

Fig. 7.—Section of stomach wall of normal guinea-pig. Shows many bacteria, perhaps spores. × 1130.

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ENTERED AS SECOND-CLASS MATTER AUGUST 1, 1911, AT THE POSTOFFICE AT CHICAGO, ILLINOIS, UNDER THE ACT OF CONGRESS, MARCH 3, 1879. ACCEPTANCE FOR MAILING AT SPECIAL RATE OF POSTAGE PROVIDED FOR IN SECTION 1103, ACT OF OCTOBER 3, 1917, AUTHORIZED ON JULY 5, 1918.

THE EXAMINATION OF SPOILED CANNED FOODS

2. CLASSIFICATION OF FLAT SOUR, SPOILAGE ORGANISMS FROM NONACID FOODS

E. J. Cameron and J. R. Esty

From the Research Laboratory, National Canners Association, Washington, D. C.

During the past 6 years there have been submitted to this laboratory for bacteriological examination 5,594 cans of spoiled and sound non-acid † products, representing 439 samples of sources of inquiry. These have been studied according to the methods suggested by Esty and Stevenson.[1] The condition of these samples has been diagnosed as sound or spoiled, and if spoiled, whether spoilage has been due to leakage or underprocessing. Cultures from underprocessed material have been kept in conservation mediums at ice box temperature. The factor of pressure cooking limits these organisms to spore forming types.

As a preliminary to intensive field work it has been considered expedient to study organisms recovered both from sound cans and those spoiled as a result of understerilization. The aim has been to establish the significance of various groups or types of organisms according to the frequency with which they have been isolated from canned foods and according to their ability to produce typical spoilage on reinoculation.

The experience of this laboratory has been [1] that when canned foods spoil as a result of understerilization, "swells" result from anaerobic activity and "flat sours" denote the presence of facultative anaerobic types. Practically the only date available concerning the relative frequency of these two types of spoilage is contained in the record book of the laboratory, and the results from our experimental packs of normal uninoculated control cans put up in connection with our sterilization studies.

Commercial experience does not afford reliable data concerning the relative frequency of loss from understerilization and leakage and

Received for publication, April 5, 1926.

† The term "non-acid" is not used here in a strict sense, but includes vegetable products whose P_H value is sufficiently high to permit the ready germination and propagation of spore forming organisms. Tomatoes, fruits and kraut are excluded.

[1] J. Infect. Dis., 1925, 36, p. 486.

nothing more than a rough estimate may be given on this basis. It should be borne in mind that figures on flat sour spoilage may be low, due to lack of external signs and consequent difficulty of detection. On the other hand, swells may be the result either of understerilization or of defective containers and in the majority of cases the swelling of occasional cans can be attributed to the latter. This is clearly shown in table 1, which summarizes the spoilage troubles incorporated in our record book. An analysis of this data also indicates that organisms causing flat sours are worthy of study as a problem of the industry, and that they are at least as important as the anaerobic types causing swells.

TABLE 1

THE RELATIVE FREQUENCY OF THE OCCURRENCE OF SWELLS AND FLAT SOURS IN UNDERSTERILIZED NONACID CANNED FOODS

Product	Number* Samples Examined	Number Samples Spoiled	Spoilage Data, in Percentages: Understerilization: Flat Sour	Understerilization: Swell	Spoilage Through Leakage
Corn	177	119	26.8	26.8	46.4
Peas	69	41	19.5	34.2	46.3
String beans	39	14	35.7	14.3	50.0
Beets	26	6	0	0	100.0
Spinach	25	15	20.0	26.7	53.3
Milk	20	10	80.0	20.0	0
Squash and pumpkin	19	13	84.6	0	15.4
Sweet potatoes	16	3	66.6	0	33.4
Pork and beans	12	3	33.4	0	66.6
Hominy	10	6	66.6	33.4	0
Miscellaneous (lima beans, succotash, asparagus)	26	11	45.4	9.2	45.4
Total	439	241	32.8	23.6	43.6

* A variable number of cans comprised each "sample."

The typical flat sour condition is that of a nongaseous acid fermentation with odor very slight or entirely lacking. Ease of detection upon tasting varies according to the normal acidity of the product involved. Thus soured corn is more readily perceptible than string beans or asparagus which may be considered in the nature of semi-acid products. The can gives no external sign of the condition of the contents, the ends remaining flat. Starchy products, of which corn is an example, may become sloppy and this condition indicated upon shaking, or a loss of vacuum may be noted upon tapping but the only certain test is that made on the can contents, preferably by means of indicators,[1] which should denote a P_H not markedly above 5.0.

There have been numerous contributions to the literature relative to the general bacteriological condition of spoiled and merchantable canned goods. Preeminent among these in extent are the works of Weinzirl,[2] Cheyney[3] and Savage.[4] Weinzirl made bacteriological examination on 1,018 samples of canned foods of which 169 were spoiled or suspected. His procedure was designed to draw a much needed microbiological picture of a wide variety of canned foods, both spoiled and sound. Weinzirl concludes that commercial canned foods as found in the market are not always sterile, but may contain viable spores of bacteria usually of the mesentericus types, unable to grow in the food due to the absence of oxygen but that these spores may develop upon admission of oxygen and spoilage result, thus making the maintenance of vacuum an essential factor in the preservation of these foods. The spore formers were practically the only organisms present due to their superior resistance to the sterilizing process.

Cheyney sought data relative to the bacteriological condition of sound canned food in a study of 725 merchantable samples. He reported that 8% of these cans contained living organisms which constitute mainly a sharply limited group of resistant spore-bearers including the B. subtilis-mesentericus group and the related thermophiles.

Savage's work has been exceedingly broad and a general review of his findings is not within the scope of this paper. In general his results on the comparative non-sterility of sound samples are confirmatory of those reported by Weinzirl, Cheyney and others. He has evidently failed, however to appreciate the importance of nongaseous acid fermentation in canned vegetables, as is evidenced by his statement: "The property of producing acid but without gas formation is of importance as regards condensed milk but otherwise is not of significance." We are unable to concur in this statement as the existence of nongaseous acid fermentation in vegetable products has been firmly established. Flat sours at one time constituted the most serious form of spoilage in canned corn and peas. Better methods of processing and cooling have, however, brought great improvement in this direction. The reader is referred to these papers for an historical and bibliographic review of the literature on the general subject.

Wyant and Tweed[5] have studied the flora of 3 samples of flat sour cold packed canned peas. This study was not sufficiently broad to permit general conclusions and it is questionable whether the flora pictured would represent the same condition with commercially packed products. The heat resistance figures for individual organisms are given and in general these are so low as to be of no significance in present commercial canning practice.

Two hundred and fourteen cultures of spore-forming, nongasforming bacteria were studied. On the basis of temperature requirements, these cultures were separated into those growing, at 37 C. but not at 55 C., at 37 C. and 55 C., and at 55 C., but not at 37 C. These have been considered as mesophiles, facultative thermophiles, and obligate thermophiles, respectively. Following this separation, the descriptive chart of the Society of American Bacteriologists was used in detailing brief descriptions of individual types. This served as a basis for

[2] J. Med. Research, 1919, 39, p. 349.
[3] Ibid., 1919, 40, p. 177.
[4] Canned Foods in Relation to Health, 1923.
[5] Agricult. Exper. Sta., Michigan Agricultural College, Technical Bulletin No. 50, 1923.

further subdivision, and certain outstanding groups were subjected to detailed study both as regards points of classification and also their ability to reproduce the flat sour condition in various foods. The recommendations of the Society of American Bacteriologists [6] with few modifications, were followed throughout this work.

After grouping in the manner described there were found to be 61 mesophiles, 98 facultative thermophiles and 55 obligate thermophiles.

Representative members of Group 80. (Facultative Thermophilic.)

Fig. 1.—Culture 1344. Group 80 isolated from flat sour baked beans. Plain agar, 24 hours at 55° C. Gram stain × 1000.

Of the 61 mesophilic cultures, 37 were found to be of the aerobic spore-forming type, the characteristics of the majority of which may be indicated by the index number, 5111-51120-1232, and the others deviating in never more than one characteristic. This evidence was further fortified by characteristic growth on potato, and in most cases,

[6] Manual of Methods, Soc. Am. Bacteriologists.

peptonization of milk. For reasons which will be discussed later in this paper these aerobic spore-forming organisms were not considered characteristic as causative agents in the spoilage of canned foods and it was not considered within the scope of this investigation to establish fine points of classification. The remaining 24 mesophilic cultures comprised a miscellaneous group of organisms possessing widely varying characteristics. Here the infrequency of recovery of any one type

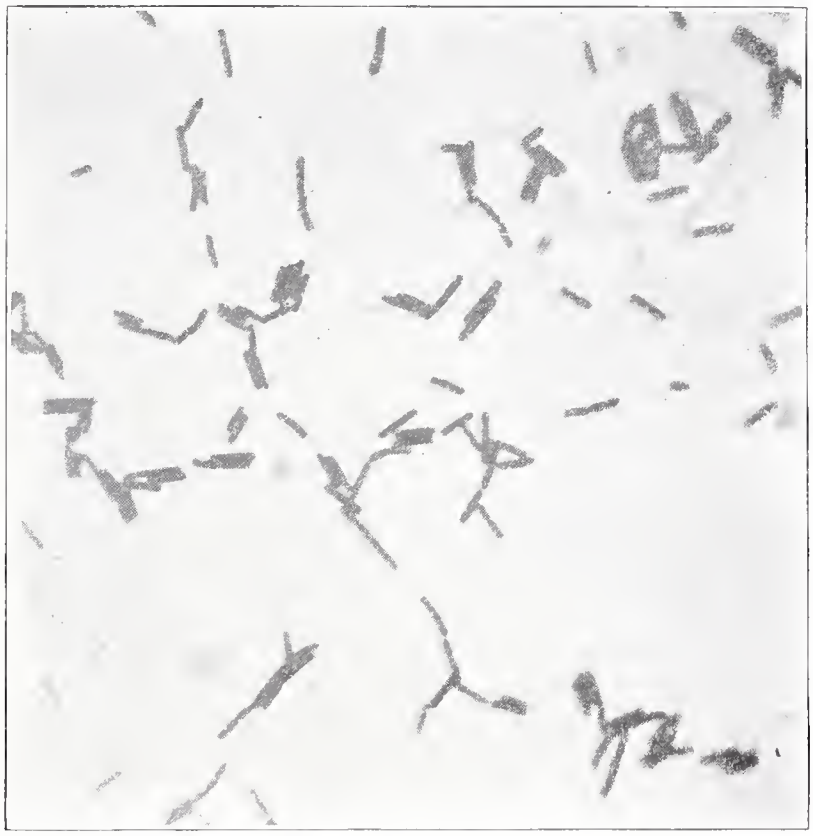

Fig. 2.—Culture 1608. Group 80. Isolated from flat sour pumpkin. Plain agar, 24 hours at 45° C. Gram stain × 1000.

limited any significance as a persistent agent in food spoilage. The evidence of this laboratory, therefore, tends to eliminate the mesophilic spore-forming organisms as causative agents in typical flat sour spoilage.

Of the 98 facultative thermophilic cultures, 35 proved to belong to the group of aerobic spore-formers, varying from those in the mesophilic group by essentially only their ability to grow at higher temperatures. Seven cultures possessed varying characteristics and were not

considered in detail. The remaining 56 cultures had all essential characteristics in common, produced typical flat sour spoilage in a number of different canned foods and were on this account, considered of extreme significance. These were considered in detailing group 80.

Of the 55 cultures of obligate thermophiles studied, all were capable of producing flat sour spoilage and appeared very closely related. On the basis of diastatic and gelatinolytic powers they were subdivided into four groups. Group 100, the members of which hydrolyzed starch but were unable to liquefy gelatin or blood serum, comprised 42 strains and were studied in detail.

Table 2 indicates the essential characteristics of organisms considered typical in the production of flat sour spoilage and the foods from which they have been isolated.

TABLE 2

TABLE SHOWING THE ESSENTIAL CHARACTERISTICS OF ORGANISMS CONSIDERED TYPICAL IN THE PRODUCTION OF FLAT SOUR SPOILAGE

Group Number	Number of Strains	Essential Characteristics (Index Number—Soc. Am. Bact. Descriptive Chart)	Spoiled Foods From Which Isolated
		Growth at 37 and 55 C.	
60	5	5221, 52220, 1222	Corn, peas
80	51	5221, 522(3)0*, 222(3)	Corn, peas, string beans, baked beans, lima beans, spinach, beets, pumpkin, hominy, potatoes, milk
		Growth at 55 C., but not at 37 C.	
90	9	5212, 52120, 1232	Corn, peas
100	42	5212, 522(2)0, 12(3)2	Corn, hominy, milk, peas, pumpkin, string beans
110	2	5212, 52120, 2232	Corn
120	2	5212, 52220, 2232	Corn, lima beans

* Parentheses indicate some variation in corresponding characteristics.

Nine strains of group 80 and 8 strains of group 100 were selected for detailed study.

GENERAL CHARACTERISTICS OF MAJOR GROUPS

Group 80 (Facultative Thermophiles) at 45 C.—Morphology: On plain agar straight rods with rounded ends occurring singly and in short chains and clumps. In 24 hours they range in size from about 1.0 mikron in thickness to about 4.0 mikrons in length.

Staining: Stains well with the usual dyes. Gram-positive with a characteristic mottling within the cell, giving the appearance of granulation. These granules have not been demonstrated with gentian violet, methylene blue, or carbol fuchsin. Definite numbers of gram-negative elements may be noted in old cultures.

Spores: Sporulation usually appears on plain agar after 48 hours incubation. These are terminal or subterminal, elliptical, measuring about 0.8 by 1.5 mikrons, and producing some bulging of the rod. Extended incubation is usually necessary for the observance of free spores.

Motility: The bacteria are motile in young agar cultures. Some variation between individual members as to the degree of motility.

Flagella: Polar and bipolar. Readily demonstrated by method of Plimmer and Paine.[7]

Relation to oxygen: Facultative anaerobic.

Nutrient broth: Turbidity slight or absent. No sediment.

Nutrient agar stab: Moderate growth along entire length of stab.

Nutrient agar stroke: Growth, abundant; form, beaded; elevation, convex; lustre, glistening; optical character, translucent in young cultures, later becoming opaque.

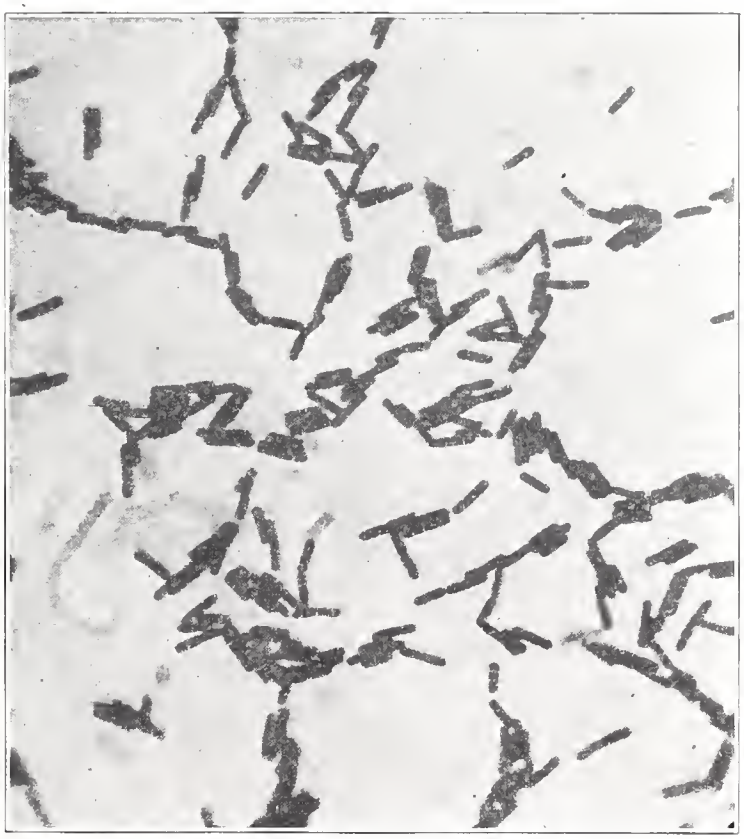

Fig. 3.—Culture 1576×. Group 80. Isolated from flat sour lima beans. Plain agar, 24 hours at 45° C. Gram stain × 1000.

Dextrose agar colonies: Well defined, circular colonies, from 2-5 mm. in diameter. Under low magnification, the colony appears distinctly granular with and without some curling. Edge, undulate. The colony is further characterized by a definite, opaque central spot.

Potato: No apparent growth.

Milk: Action variable. Some strains exert no apparent influence on the reaction. Others produce acid, usually with curdling. No evidence of gas. No peptonization.

[7] J. Path. & Bact., 1921, 24, p. 286.

Nitrate broth: Not reduced.

Blood serum and gelatin: No liquefaction.

Catalase: Positive.

Starch agar: Hydrolysis negative or very slight.

Pathogenicity: Nonpathogenic.

Temperature relations: On nutrient agar. Maximum temperature for growth, 65 C. Minimum temperature for growth, 30 C.–35 C. Some strains show slight growth at 25 C. Optimum temperature, 45 C.–50 C.

Fermentation reactions: Action in carbohydrates with suggestions as to the possibility of subgrouping on the basis of carbohydrate reactions, may be noted by referring to table 3. The amount of acid formed and the percentage of carbohydrate consumed were not determined, notations being made on the basis of change in indicator. Glucose, galactose, fructose and mannose were readily fermented by all strains tested. Some variation was noted in the amount of acid produced by individual strains in the pentose broths. Seven of the eight strains produced acid from maltose and lactose, while only three were able to utilize sucrose. None of these strains were able to utilize inulin, cellulose or glycerol.

Acid produced in:	No acid produced in:	Action variable in
Xylose	Inulin	Rhamnose
Arabinose	Cellulose	Sucrose
Glucose	Glycerol	Maltose
Galactose		Raffinose
Mannose		Starch
Fructose		Mannitol
		Lactose
		Salicin

Group 100 (Obligate Thermophiles) at 55 C.—Morphology: On plain agar straight rods with rounded ends, occurring singly, in short chains and clumps. In 24 hours they range in size from about 0.7 mikrons in thickness to about 3.5 mikrons in length.

Staining: Stains well with the usual dyes. Gram-negative with or without the appearance of few minute gram-positive granules, acetone-alcohol used as decolorizing agent.

Spores: Sporulation usually appears on plain agar in 24 hours. These are terminal or sub-terminal, elliptical, measuring about 1.2 by 2.0 mikrons, producing some bulging of the rod. Elliptical free spores may be observed in 48 hours measuring on the average 1.2 mikrons by 2.5 mikrons.

Motility: Motile in young agar cultures.

Flagella: Peritrichic. Readily demonstrated by the method of Plimmer and Paine.[7]

Relation to oxygen: Facultative anaerobic.

Nutrient broth: Moderate turbidity, flocculent sediment, no odor.

Nutrient agar stab: Moderate growth along entire length of stab.

Nutrient agar stroke: Growth, abundant; form, beaded; elevation, convex; lustre, glistening; optical character, opaque.

Dextrose agar colonies: Well defined, circular colonies, from 2–5 mm. in diameter. Under low magnification the colony appears granular with no curling. Edge undulate. The colony has also the peculiar central spot noted in group 80.

Potato: No growth.

Milk: Rennet curd usually formed after 5–7 days' incubation.

Nitrate broth: Slight reduction.
Blood serum and gelatine: No liquefaction.
Catalase: Positive.
Starch agar: Extensive hydrolysis after 1–2 days' incubation.
Pathogenicity: Non-pathogenic.
Temperature relations: On nutrient agar. Maximum temperature for growth, 72.5 C. Minimum temperature for growth, 42 C. to 45 C. Optimum, 55 C. to 60 C.

Representative members of Group 100 (Obligate Thermophilic).

Fig. 4.—Culture 1503. Group 100. Isolated from flat sour peas. Plain agar, 24 hours at 55° C. Gram stain × 1000.

Fermentation reactions: The action in carbohydrates of individual members of group 100, is shown in table 3. Glucose, galactose, mannose, sucrose, maltose and starch were readily fermented with the production of acid by all strains tested. None of the strains fermented rhamnose, inulin, cellulose or mannitol. Variations were noted in the ability of these strains to produce acid from xylose, arabinose, fructose, lactose, raffinose, salicin and glycerol.

TABLE 3

FERMENTATION CHARACTERISTICS OF INDIVIDUAL STRAINS

Reaction in 48 hours, unless otherwise noted, in 1% of the carbon compound in standard nutrient, 1% difco peptone broth, P_H approximately 7.0, with Brom Cresol Purple as indicator.

	Sub Number	Food from which Isolated	Acid Produced in																
			Xylose	Arabinose	Rhamnose	Glucose	Galactose	Mannose	Fructose	Sucrose	Maltose	Lactose	Raffinose	Starch	Inulin	Cellulose	Salicin	Mannitol	Glycerol
	2547	Corn.....	+	+	0	+	+	+	+	0	+	+	0	0	0	0	0	+ 7 days	0
	1460	Peas.....	+	+	+	+	+	+	+	0	+	+	0	0	0	0	+ 7 days	+ 7 days	0
	4578	String beans...	+	+	+	+	+	+	+	0	+	+	0	0	0	0	+ 7 days	+ 7 days	0
	1576	Lima beans....	+	+	+ 7 days	+	+	+	+	0	+	+	0	0	0	0	+ 7 days	+ 7 days	0
Group 80	1734	Beets.....	+ Weak	+ Weak	0	+	+	+	+	+	+	+	+ 7 days	+ 4 days	0	0	+	0	0
	1590	Spinach.....	+ Weak	+ Weak	0	+	+	+	+	+	+	+ Weak	+ 7 days	+ 7 days	0	0	0	0	0
	1608	Squash.....	+	+ Weak	0	+	+	+	+	0	+	+	0	+ 7 days	0	0	0	0	0
	1215	Hominy.....	+	+	0	+	+	+	+	0	+	+	0	+ 7 days	0	0	0	0	0
	1344	Baked beans...	+ Weak	+ Weak	0	+	+	+	+	+ 5 days	0	0	0	0	0	0	0	0	0
	1792	Corn.....	0	+	0	+	+	+	+	+	+	0	+	+	0	0	0	0	+ 3 days
	E.B.17-H-1	Corn.....	0	+	0	+	+	+	+	+	+	0	+	+	0	0	0	0	+ 7 days
	1592	Hominy.....	+ 3 days	+	0	+	+	+	+	+	+	0	+	+	0	0	0	0	+ 3 days
Group 100	1503	Peas.....	+ 3 days	+	0	+	+	+	+	+	+	0	+	+	0	0	0	0	+ 3 days
	1492	Pumpkin.....	+	+	0	+	+	+	+	+	+	0	+	+	0	0	0	0	0
	1476	Milk.....	0	+ 3 days	0	+ 3 days	+ 3 days	+ 3 days	0	+	+	+	0	+	0	0	+	0	0
	1574	Peas.....	0	+	0	+	+	+	+	+	+	+	+	+	0	0	+	0	0
	1376	Hominy.....	0	0	0	+	+ 3 days	+	+ 3 days	+	+	0	+ 3 days	+	0	0	0	0	+ 7 days

HEAT RESISTANCE

Bigelow and Esty [8] have studied thermophilic flat sour organisms from the standpoint of heat resistance and herein lies an explanation as to their ability to survive the canning process. Data incorporated in table 4 is taken from their published account of this work. Organism 1608 is representative of group 80, while 1476, 1492, 1503 and 1592 are representative of group 100.

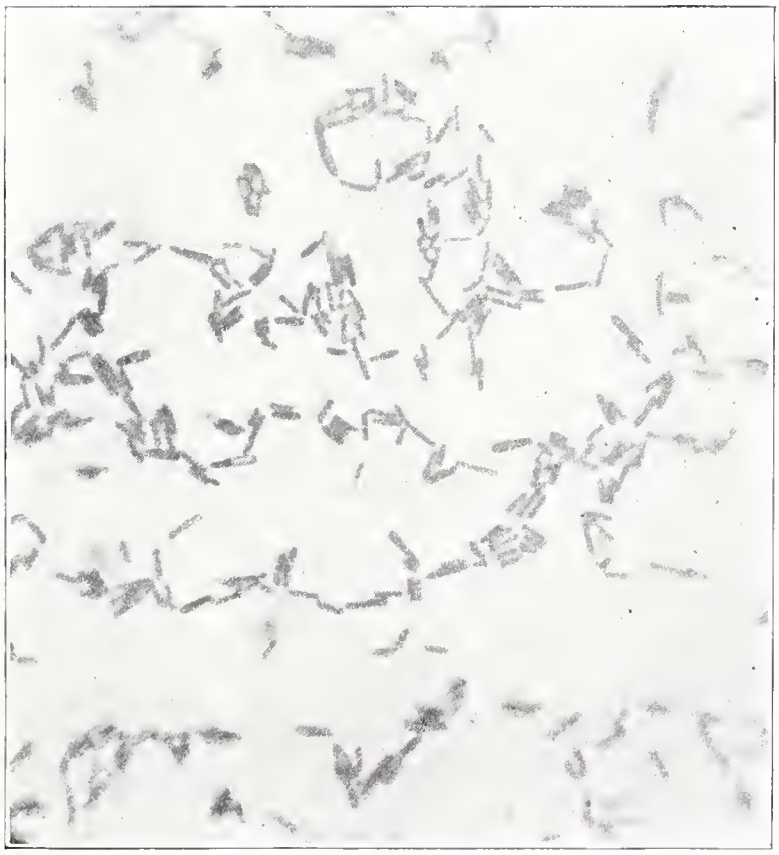

Fig. 5.—Culture 1356. Group 100. Isolated from sweet hominy. Plain agar, 24 hours at 55° C. Gram stain × 1000.

NOMENCLATURE

Literature search has failed to reveal any described thermophile which might be closely identified with our facultative thermophilic group 80. The nearest approach is provided in Bergery's descriptions of B. thermotranslucens and B. thermoliquefaciens.[9] These may be eliminated, however, on the basis of possession of peritrichous flagella

[8] J. Infect. Dis., 1920, 27, p. 602.
[9] Manual of Determinative Bacteriology, 1923.

and slight growth at 37 C. We refrain from naming this type as it is possible that there may be a much closer relation with previously described types than is apparent by comparison with written descriptions, and we desire to avoid adding to the confusion which already exists in the literature through duplication of names.

With the exception of observations as to motility and the presence of flagella, strains in group 100 are identical with the thermophilic organism described by Donk [10] and named by him B. stearothermophilus. Due to this discrepancy the culture described by Donk was revived and studied with reference to its motility and possession of flagella. Motility was at all times sluggish but was definite in young cultures after a series of transfers. Flagella were eventually demonstrated by

TABLE 4

Time Necessary to Destroy the Spores of Typical Thermophiles at Temperatures Stated *

Culture Number	Number of Bacterial Spores per Cc.	Corn Juice P_H 6.1	Minutes Required to Destroy Spores in Corn Juice P_H 6.1 at 100 C.		105 C.		110 C.		115 C.		120 C.	
			+	—	+	—	+	—	+	—	+	—
1608	12,000	6.1	210	240	95	100	30	32	4	5	2	3
1476	70,000	6.1	750	780	380	400	110	115	33	36	9	10
-1492	225,000	6.1	789	840	360	390	100	105	35	38	10	11
1503	100,000	6.1	1020	1080	500	525	170	175	55	58	16	17
1592	115,000	6.1	1140	1200	575	600	185	190	66	70	18	19

* From Bigelow and Esty [8]: "The Thermal Death Point in Relation to Time of Typical Thermophilic Organisms."

the method of Plimmer and Paine but no success followed the use of the Muir modification of Pittsfield's method.

Groups 90, 110 and 120 vary from group 100 essentially in their action on starch and gelatin and in view of the otherwise close agreement, it is not felt that these differences would justify their designation as individual species, since the methods used in determining these characteristics were not entirely satisfactory in defining strict differentiations at high temperatures.

DISTRIBUTION

Table 5 shows the occurrence of the two major flat sour groups among the cultures studied. Their distribution is indicated by noting the various states from which flat sour foods were sent to this laboratory for examination. It is to be noted that the presence of these bacterial types is not peculiar to any restricted part of the United States.

[10] J. Bact., 1920, 5, p. 373.

GROWTH IN FOODS

Six strains of aerobic spore formers, three mesophilic and three facultative thermophilic, were used in inoculation experiments in corn, peas, string beans, evaporated milk and spinach. These were incubated for seven days at 37 C. and from two to five days at room temperature.

Fig. 6.—Culture 1792. Group 100. Isolated from flat sour corn. Plain agar, 24 hours at 55° C. Gram stain × 1000.

One set was held approximately 200 days at room temperature, before testing for spoilage. The cans were punched, inoculated and soldered under conditions as nearly aseptic as circumstances permitted. The unavoidable entrance of air permitted in this operation must be con-

sidered as an experimental disadvantage. Upon examination after incubation of the foods inoculated the following results were obtained.

None of the strains tested had any perceptible effect on string beans, milk and spinach. One of the 6 strains produced an undesirable off taste in canned corn, and two strains, including that held 200 days, produced slight souring in peas, perceptible only upon colorometric tests. The organism was recovered in every case but smears from cans, including the off samples, failed to show perceptible vegetation. We have been unable to explain the tainted condition of these three cans in the absence of growth by the aerobic spore formers.

TABLE 5

SHOWING OCCURRENCE OF THE TWO MAJOR FLAT SOUR GROUPS AND INDICATING THEIR WIDE DISTRIBUTION

Group	Food	Occurrence Among Cultures Studied	States in Which Spoilage Occurred
80	Corn	15	Illinois, New York, Maryland, Tennessee, Iowa
	Peas	8	New York, Wisconsin, Illinois
	String beans	5	Maryland, New York, Texas, Virginia
	Pumpkin	6	New Jersey, Michigan, Illinois
	Spinach	5	Michigan, California
	Irish potatoes	1	Michigan
	Beets (isolated from mixed cultures)	2	New York
	Baked beans	2	New York
	Milk	3	Pennsylvania, Wisconsin
	Lima beans	2	Michigan
	Hominy	2	Illinois
100	Corn	25	Indiana, New York, Iowa, Ohio, Illinois
	Hominy	8	Iowa, Illinois, Colorado
	Peas	6	Utah, New York, Illinois
	Pumpkin	1	Illinois
	Milk	1	California
	String beans	1	Tennessee

The facultative thermophilic group 80, considered typical in the production of flat sours has been found to be able to produce acid in corn, peas, hominy, potatoes, lima beans, baked beans, string beans, spinach, beets, pumpkin, milk, broccoli and asparagus, reducing the P_H in all cases to below 5.0, and in most instances below 4.5.

The obligative thermophilic group 100, has been found consistently capable of producing flat sours in corn, peas and broccoli, the lower limit of P_H being about 5.0. Certain strains produce acid in evaporated milk and string beans while others do not. Members of this group possess the contradictory property of producing an intensely sweet taste in canned hominy, the result being known as sweet hominy; this by virtue of the exo-cellular starch splitting enzymes. This condition is rare but

in every case known to this laboratory it has been possible to isolate a member of group 100. Reducing sugars have been found even as high as 9% in the hominy liquor although originally absent. Maltose greatly predominates over glucose. The P_H (6.3–6.5) does not vary radically from the normal of about 7.0. A study of this condition indicates that the thermophilic bacteria develop as a result of faulty cooling procedure and give rise to the starch splitting enzymes with amounts of acid varying according to the length of time the temperature of can is within limits for growth. The organism is rendered inactive when the can cools and acid production ceases but the diastatic activity, although inhibited, continues to function over an indeterminate period to produce the characteristic sweetness. Why this condition is not met with in canned corn can only be surmised, but it is felt that here the enzymes of the organism function normally, while in hominy the lye treatment to which this product has been subjected may operate to produce a metabolic disturbance resulting in abnormal end products.

DISCUSSION

Previous studies relative to the bacteriological condition of sound canned foods have almost invariably resulted in the opinion that such foods are not universally sterile and that aerobic spore formers are to be expected, among the isolated organisms. Although no figures are available regarding the extent of nonsterility, the same is the experience of this laboratory. That the presence of organisms of this type is not a serious cause of unsoundness in canned foods is the consensus of opinion, and with this we are in complete accord. Weinzirl [2] minimizes the presence of aerobic spore formers as potential spoilage agents but suggests that they are the usual causes of spoilage when cans are defective and leak. This is not in accord with the experience of this laboratory. Bushnell [11] considers B. mesentericus and B. subtilis to predominate in unspoiled canned foods and as a result of studies of these organisms in sealed tubes concludes that small amounts of air allow only minimal growth. B. mesentericus was found to be less affected by the absence of air than B. subtilis. Savage [4] being unable to completely prevent the growth of these types under the strictest anaerobic conditions he could devise, concluded that the aerobic spore formers possessing proteolytic properties were potential causes of decomposition of canned foods. We have been unable in this laboratory to correlate proteolytic

[11] Ibid., 1922, 7, p. 283.

decomposition in canned foods with the presence of aerobic spore formers and our experimental data would suggest such a happening to be so rare as to be of no significance.

Wyant and Tweed [5] have studied "organisms resembling aerobic spore-forming soil organisms" with reference to their ability to produce flat sours in canned products and conclude that these bacteria may be responsible for such a condition. A P_H of 5.6 in pea juice represents the limit of the acid producing powers of these types and this does not differ greatly from the normal (6.2–6.4). Although they are flat sour in a strict sense, the conception of the industry requires a more marked acidity than is thus indicated. Here, the term, flat sour, is applied only to spoiled products with a distinctly sour taste. This calls for a P_H value not markedly above 5.0.

The group of obligate thermophiles considered in this paper have shown no visible growth or activity evidenced by measurable P_H changes below 40 C. Spores of several typical strains inoculated into tubes of dextrose broth and corn juice have demonstrated no apparent activity during an incubation of over five years at 35 C.–37 C., yet cultures transferred to a temperature of 55 C. will show good growth within 16 hours. Canned corn, containing viable spores of one thermophile, has been held at 22 C. and 37 C., during three years without change. Incubation of some of this material at 55 C., however, invariably results in proliferation and acid production. It may be inferred that Morrison and Tanner [12] doubt the existence of types unable to grow below 40 C. in the statement, "it is possible that statements by several investigators that the thermophilic bacteria would not grow at room temperature may have been made without waiting a sufficient period for growth to appear." Considerable time has been spent during the course of the present investigation in attempting to produce growth at 37 C. in a variety of mediums aerobically and anaerobically. Failure has resulted. A close study of Morrison and Tanner's work results in the opinion that these workers were considering a thermophilic division of the aerobic spore forming soil organisms and did not consider in detail any types even remotely related to group 100. Bacteria of the sort described by these workers have been encountered during the present investigation. While not so resistant to heat as the thermophiles considered here, they possess a relatively high resistance to heat and may resist a process when present in large numbers. They are widely distributed but, due to their oxygen requirements are not significant in the spoilage of canned foods.

[12] Botanical Gazette, 1924, 77, No. 2.

SUMMARY

The 214 cultures of nongas-forming, spore-forming bacteria, isolated from spoiled and sound canned foods were studied to determine types significant in the production of flat sour spoilage.

Two large thermophilic groups were defined as causing this type of spoilage. Group 80, a facultative thermophilic group, comprised 51 cultures and had been isolated from pure cultures in corn, peas, string beans, baked beans, lima beans, spinach, milk, pumpkin, hominy and potatoes; and from mixed cultures in beets. Group 100, an obligative thermophilic group comprised 42 cultures and had been isolated from pure cultures in corn, hominy, milk, peas, pumpkin and string beans.

Upon reinoculation it was found that group 80 produced the typical flat sour condition in corn, peas, hominy, potatoes, lima beans, baked beans, string beans, spinach, beets, pumpkin, milk, broccoli and asparagus. Group 100 produced flat sours in corn, peas and broccoli and by virtue of starch splitting enzymes, sweetness in hominy. Certain strains could sour evaporated milk and string beans.

STANDARDIZATION OF TYPHOID VACCINE BY PHOTOMETRIC METHODS

Adelaide B. Baylis

From the Department of the Laboratories, New York Post-Graduate Medical School and Hospital

Owing to an inquiry from Dr. J. W. Smith, Jr., of the Army Medical School, as to the suitability of the photometer in estimating the strength of bacterial suspensions, we have undertaken a series of investigations along this line. The lack of precision in the commonly used Wright's method of counting and in the various other procedures employed in standardizing bacterial vaccines has been the general experience of workers in this field. Furthermore, all counting methods are time consuming and tempt the worker to make his estimation without counting an adequate number of organisms. Since the completion of our investigations Smith [1] has published two articles on this subject and from our own experience we are in accord with his critical statements in regard to the accuracy of such counts. In addition we would emphasize the error of the erythrocyte count generally used for comparison, the standard of five million red blood cells per cmm. being the figure generally used. This is the normal count for man and as the majority of laboratories employ women, even if the women had a normal cell count it would lead to a 10% error. Rarely is the cell count up to normal—as for example, three workers on vaccines in our own laboratory had their erythrocyte count made as a matter of verifying this point. The result revealed that had they made their calculations on the basis of the usual standard of 5,000 million per cc. there would have been respective errors of 7.92%, 10.56% and 13.44%.

In the preparation of an autogenous vaccine to be injected into only one individual, great precision in standardizing the strength is not required, because it is customary to begin with a relatively small dose and to increase it, guided by the response of the patient. When, however, stock vaccines are prepared for administration to several or to many individuals, either for treatment or for immunization, the need of at least reasonable precision in standardization is generally recognized and requires no discussion.

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[1] Am. J. Pub. Health, 1925, 15, p. 433; J. Infect. Dis., 1925, 37, p. 385.

The present communication presents comparative results on ten bacterial suspensions made from different cultures of the typhoid bacillus, the strength of suspension being estimated, first, by the microscopic counting method of Wright on dry film, second by the reading of the absorption of light in the photometer and third by the reading of the diffusion of light in the same instrument. The data of the observations are presented in tabular form and the maximum, the minimum and the selected mean readings at each dilution are represented in graphs.

The typhoid bacillus was grown for 24 hours on agar slants at 37 C. The growth was microscopically examined and then emulsified with sterile 0.9% saline, and the emulsion centrifugalized for twenty minutes at high speed. The washing and centrifugalization was carried out three times, and after the final washing the suspension was sealed in an ampule and sterilized for one hour in a water bath at 58 C. The actual count was made at this stage so that any lysis caused by sterilization would not introduce an error in the count. Then sufficient sterile saline with 0.25% phenol was added to make a dilution of 5,000 million organisms for 1 cc. of vaccine.

For the count itself three blood slides were made and a count of two hundred cells and organisms made from each. If the three counts were close to each other then their average was taken and a dilution of 5,000 million per 1 cc. of vaccine made in accordance with this average. If the counts varied to any appreciable amount the slides were discarded and new slides made. In the later part of our experiments another check was introduced into the technic by counting two blood slides made from the dilution of 5,000 million. The stock suspension was then placed in a special bottle and stored in the refrigerator until made up to the required dilutions with sterile salt solution. Sterile precautions were observed throughout and where there was a question of technic or photometric error in estimation the particular tube in question was discarded. Agar slants were made and incubated at the end of sterilization to ascertain if all growth had been killed and also at each time when the stock solution was withdrawn for photometric readings. The bottle for the storage of the stock suspension is one devised to prevent as far as possible any contamination and at the same time permit easy removal of any desired amount of suspension without opening the bottle. It consists of a wide-mouthed bottle with a rubber stopper, held firmly in place by wire bands. Two glass tubes are run through this stopper, one (A) reaching to the bottom of the bottle, the other (B) just below the bottom

of the stopper. To the portion of each of the tubes above the stopper is attached a short piece of rubber tubing with glass bead insert and to this tubing on (A) a short pipette and on (B) a short piece of glass tubing—this well packed with cotton as an air filter. The bottle is sterilized in an oven to 200 C and the cork and glassware in the autoclave for a half hour at 20 pounds. At the time of withdrawing the stock vaccine for dilution, the bottle is shaken vigorously and a constant pressure bulb attached to (B), the rubber tubing is compressed at the

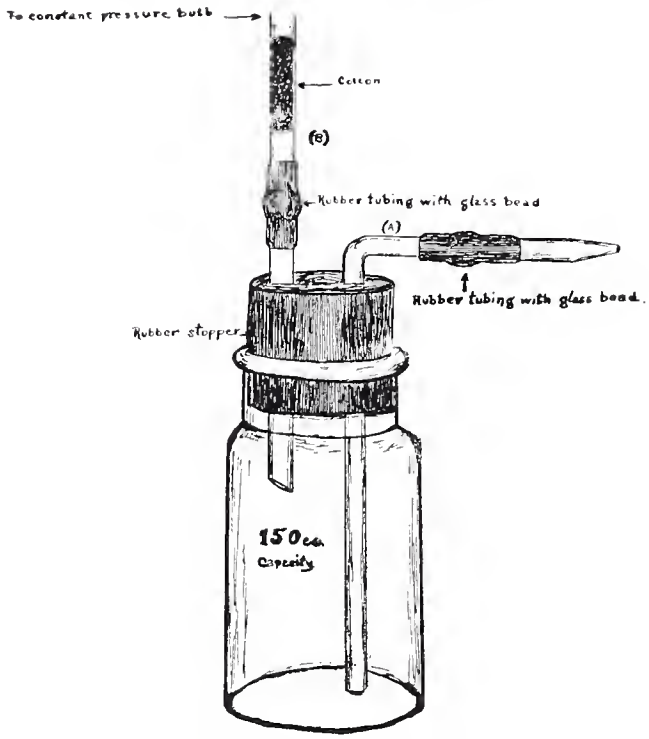

Fig. 1.—Storage bottle for stock suspension.

point of the glass bead and air introduced into the bottle. The bottle is again shaken vigorously, the rubber tubing attached to (A) compressed at the point of the glass bead and the necessary amount of stock vaccine allowed to flow out.

The photometer used is the photométre of Vernes, Bricq and Yvon, devised by them for use in the Vernes flocculation test for syphilis. A detailed description of this delicate and precise instrument has been given in an article by Baylis, Sheplar and MacNeal.[2] Briefly it is a type of nephelometer fitted with lenses, prisms, eyepieces and an adjustable

[2] Am. J. Syphilis, 1926, 10, p. 298.

wedge of fumed glass attached to a scale for direct reading. There are two sets of diaphragms or surcharges to enable the regulation of the amount of light according to the density of the fluid to be estimated. The photométre may be used for the estimation of either absorption or diffusion of light and the results obtained are recorded in mg. per 1 cc. of fluid. The makers supply with each instrument the necessary figures for the calculation for the amount of light intercepted by the surcharges, and this calculation is only necessary when different surcharges are employed. Before making the recorded observations it was thought advisable to test the technic and the adaptability of the photométre for the estimation of vaccines. This test was carried out in the following manner. A stock suspension of paratyphoid bacillus of 5,000 million organisms per cc. was used to prepare four dilutions, 500 million, 1,000

TABLE 1

EXPERIMENTAL READINGS, BACILLUS PARATYPHOSUS A

Tube	Dilution, Millions per cc.	Actual Readings		Difference	Calculated Results		Difference
		1	2		1	2	
		Absorption					
1	500	1.43 O/A	1.43 O/A	0	0.29 O/O	0.29 O/O	0
2	1,000	1.63 O/A	1.62 O/A	0.01	0.49 O/O	0.48 O/O	0.01
3	3,500	1.35 O/O	1.34 O/O	0.01	1.35 O/O	1.34 O/O	0.01
4	5,000	1.87 O/O	1.87 O/O	0	1.87 O/O	1.87 O/O	0
		Diffusion					
1	500	2.30 B/O	2.33 B/O	0.03	5.2170	5.2497	0.0327
2	1,000	2.10 B/O	2.10 B/O	0	4.9990	4.9990	0
3	3,500	1.56 B/O	1.56 B/O	0	4.4094	4.4094	0
4	5,000	1.48 B/O	1.45 B/O	0.03	4.3232	4.2905	0.0327

million, 3,500 million and 5,000 million, each dilution prepared in duplicate and placed in small test tubes. The tubes were then marked by an associate with letters chosen at random with no possible order of sequence and the markings recorded with the dilutions they represented. The duplicate set was then given to the operator for estimation, both for absorption and diffusion of light and the results of the readings recorded opposite the letter of the tube. After all the readings had been made the markings were sorted out by the associate and not until then did the operator have any idea of the dilutions that the tubes contained. The remarkable results as shown by table 1 gave assurance as to the accuracy of the photométre and the technic, justifying the continuation of the test.

The tables represent the actual reading of the photométre with surcharges employed and the calculated results per 1 cc. of fluid. For

TABLE 2

EBERTHELLA TYPHI—ABSORPTION

Tube	Dilution, Millions per Cc.	Selected Reading	Actual Readings 1	2	3	4	5	6	7	8	9	10
1	500	1.43 O/A	1.47 O/A	1.38 O/A	1.43 O/A	1.43 O/A	1.43 O/A	1.45 O/A	1.43 O/A	1.41 O/A	1.43 O/A	1.43 O/A
2	1,000	1.60 O/A	1.56 O/A	1.59 O/A	1.61 O/A	1.60 O/A	1.60 O/A	1.60 O/A	1.62 O/A	1.58 O/A	1.59 O/A	1.60 O/A
3	1,500	1.73 O/A	1.72 O/A	1.72 O/A	1.73 O/A	1.73 O/A	1.73 O/A	1.73 O/A	1.74 O/A	1.71 O/A	1.73 O/A	1.72 O/A
4	2,000	1.85 O/A	1.87 O/A	1.88 O/A	1.85 O/A	1.89 O/A	1.87 O/A	1.83 O/A	1.85 O/A	1.82 O/A	1.82 O/A	1.82 O/A
5	2,500	2.04 O/A	0.81 O/O	2.02 O/A	2.04 O/A	2.05 O/A	2.05 O/A	2.04 O/A	2.04 O/A	2.00 O/A	2.03 O/A	2.03 O/A
6	3,000	2.25 O/A	0.97 O/O	2.19 O/A	2.27 O/A	2.20 O/A	2.26 O/A	2.25 O/A	2.26 O/A	2.23 O/A	2.25 O/A	2.26 O/A
7	3,500	2.35 O/A	1.11 O/O	2.36 O/A	2.35 O/A	2.37 O/A	2.38 O/A	2.35 O/A	2.34 O/A	2.36 O/A	2.37 O/A	2.36 O/A
8	4,000	2.47 O/A	1.27 O/O	2.50 O/A	2.47 O/A	2.49 O/A	2.48 O/A	2.47 O/A	2.47 O/A	2.47 O/A	2.46 O/A	2.47 O/A
9	4,500	1.61 O/O	1.41 O/O	.....	1.61 O/O	1.61 O/O	1.61 O/O	1.61 O/O	1.61 O/O	1.60 O/O	1.60 O/O	1.61 O/O
10	5,000	1.73 O/O	1.61 O/O	1.73 O/O	1.75 O/O	.....	1.75 O/O	1.75 O/O	1.75 O/O	1.75 O/O	1.75 O/O	1.75 O/O
			Calculated Absorption (O/O)									
1	500	0.29	0.33	0.24	0.29	0.29	0.29	0.31	0.29	0.27	0.29	0.29
2	1,000	0.46	0.42	0.45	0.47	0.46	0.46	0.46	0.48	0.44	0.45	0.46
3	1,500	0.59	0.58	0.58	0.59	0.59	0.59	0.59	0.60	0.57	0.59	0.58
4	2,000	0.71	0.73	0.74	0.71	0.75	0.73	0.69	0.71	0.68	0.68	0.68
5	2,500	0.90	0.84	0.88	0.90	0.91	0.91	0.90	0.90	0.86	0.89	0.89
6	3,000	1.09	0.97	1.05	1.13	1.06	1.12	1.11	1.12	1.09	1.11	1.12
7	3,500	1.22	1.11	1.22	1.21	1.23	1.24	1.21	1.20	1.22	1.23	1.22
8	4,000	1.33	1.27	1.36	1.33	1.35	1.34	1.33	1.33	1.33	1.32	1.33
9	4,500	1.61	1.41	....	1.61	1.61	1.61	1.61	1.61	1.60	1.60	1.61
10	5,000	1.73	1.61	1.73	1.75	....	1.75	1.75	1.75	1.75	1.75	1.75

TABLE 3

EBERTHELLA TYPHI—DIFFUSION

Tube	Dilution, Millions per Cc.	Selected Reading	Actual Readings B/O 1	2	3	4	5	6	7	8	9	10
1	500	2.34	2.27	2.35	2.33	2.34	2.34	2.33	2.33	2.34	2.34	2.34
2	1,000	2.06	2.10	2.06	2.05	2.05	2.06	2.05	2.05	2.05	2.06	2.05
3	1,500	1.88	1.91	1.94	1.87	1.89	1.88	1.87	1.88	1.86	1.88	1.89
4	2,000	1.77	1.83	1.83	1.75	1.77	1.76	1.75	1.75	1.76	1.77	1.77
5	2,500	1.73	1.75	1.73	1.73	1.73	1.73	1.71	1.73	1.73	1.73	1.73
6	3,000	1.63	1.68	1.62	1.63	1.62	1.65	1.63	1.63	1.64	1.63	1.63
7	3,500	1.56	....	1.55	1.55	....	1.56	1.56	1.55	1.56	1.56	1.56
8	4,000	1.51	1.61	1.49	1.51	1.51	1.51	1.51	1.51	1.51	1.52	1.52
9	4,500	1.43	1.48	1.42	1.42	1.42	1.43	1.44	1.42	1.42	1.43	1.43
10	5,000	1.38	1.43	1.38	1.38	1.38	1.38	1.38	1.38	1.38	1.39	1.38
			Calculated Diffusion									
1	500	5.2606	5.1843	5.2715	5.2497	5.2606	5.2606	5.2497	5.2497	5.2606	5.2606	5.2606
2	1,000	4.9554	4.9990	4.9554	4.9445	4.9445	4.9754	4.9445	4.9445	4.9445	4.9554	4.9445
3	1,500	4.7592	4.7919	4.8246	4.7483	4.7701	4.7592	4.7483	4.7592	4.7374	4.7592	4.7701
4	2,000	4.6393	4.7047	4.7047	4.6175	4.6393	4.6284	4.6175	4.6175	4.6284	4.6393	4.6393
5	2,500	4.5957	4.6175	4.5957	4.5957	4.5957	4.5957	4.5739	4.5957	4.5957	4.5957	4.5957
6	3,000	4.4867	4.5412	4.4758	4.4867	4.4758	5.4085	4.4867	4.4867	4.4976	4.4867	4.4867
7	3,500	4.4094	.....	4.3995	4.3995	.....	4.4104	4.4104	4.3995	4.4104	4.4104	4.4104
8	4,000	4.3559	4.4649	4.3341	4.3559	4.3559	4.3559	4.3559	4.3559	4.3559	4.3668	4.3668
9	4,500	4.2687	4.3232	4.2578	4.2578	4.2578	4.2687	4.2796	4.2578	4.2578	4.2687	4.2687
10	5,000	4.2142	4.2687	4.2142	4.2142	4.2142	4.2142	4.2142	4.2142	4.2142	4.2251	4.2142

the estimation of absorption the readings are reduced to a common basis, that is without surcharges, so they are comparable. For the estimation of diffusion the actual photometric readings are calculated according to the surcharge employed, and the coefficient figure for the fumed wedge, thus obtaining the actual degrees of diffusion. A selected figure has been obtained from the ten readings and is recorded in the first column. When a certain reading has been in predominence this has been taken as the true figure and when there has been a difference in the readings in an equal number of instances the average of the ten readings has been used. This was thought best as, for example, in one instance where there were eight identical readings in the series of ten, had an average of the ten been made, the resulting figure would have been different from the eight identical readings. These readings would seem to be the more correct point for the natural curve and the other two readings due probably to errors in technic. In the diffusion the final calculated estimation was made from the results of the selected figures of the direct or actual readings.

In both of these tables it will be observed that the greatest variation in results is in 1, the first lot of vaccines to be estimated, and as the work progressed the readings became closer. At first it was thought best to discard this reading so that it might not lead to confusion of mind. Later, however, it was deemed wiser to retain the first result as undoubtedly the technic was not as perfect at the beginning and the variation served to illustrate how very easy it was, even under the most ideal conditions, to make an erroneous count.

The curve has been plotted from the selected readings and is represented by a solid black line, the readings by circles. The maximum and minimum readings are indicated by dotted lines on each side. In each instance the figures of the readings have been recorded on the graph.

Again we observe more clearly in this graph the variation of 1, especially at the 4,000 million dilution, where there has been an obvious error. As this has been already explained no further comments will be made. Careful examination reveals certain facts brought to light by the graphs which are not as prominent in the tables. The most important it would seem, is the greater variation shown in the graph of the absorption than in the graph of the diffusion, pointing to the fact that the absorption more readily detects any slight change and is therefore the more delicate test of the two. However it would seem wisest to use both methods of estimation, one to act as a check against the other, if

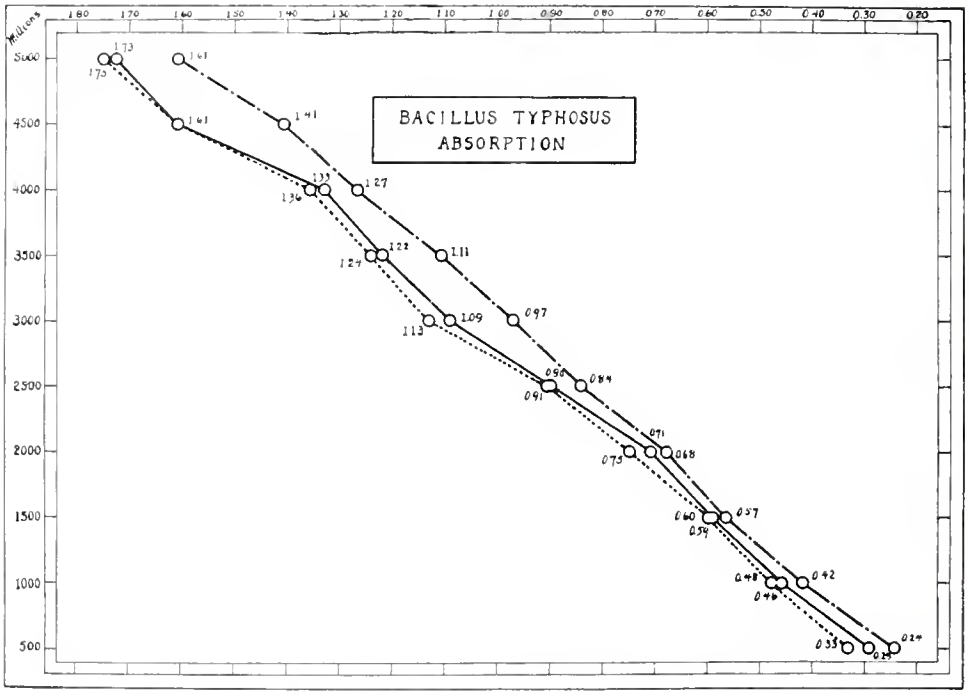

Fig. 2.—Graph of selected readings of absorption by indicated dilutions.

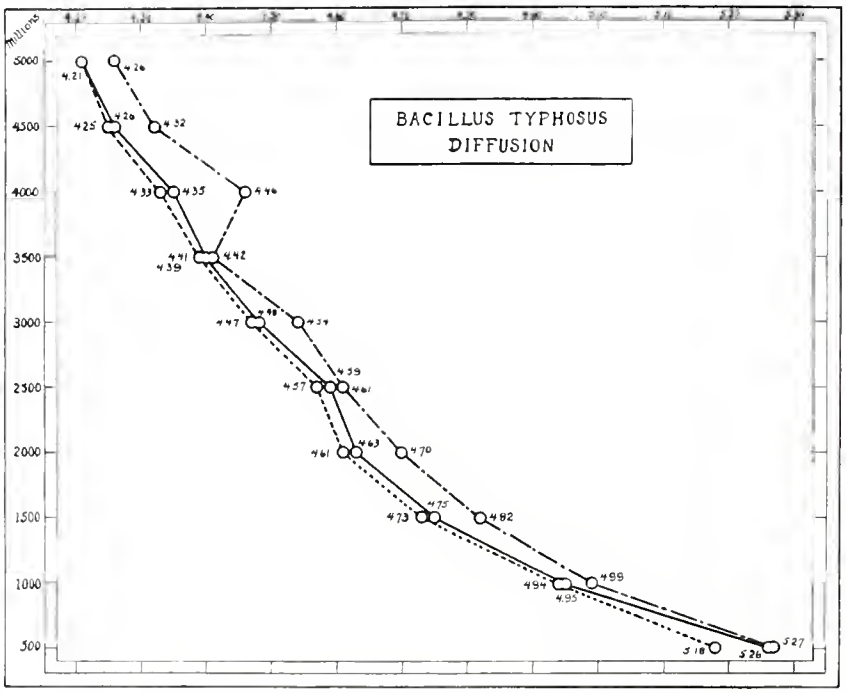

Fig. 3.—Graph of selected readings of diffusion for indicated dilutions.

this technic is to be adopted for use in the standardization of bacterial vaccines. Note also that as the number of organisms decreases, the readings become closer. As these lower readings represent the amounts generally used in the vaccines it would appear that the chance of a significant error in photometric standardization is negligible.

SUMMARY

The usual method of estimating vaccines by counting in comparison with erythrocytes offers many factors of error from the outset. When the vaccine is used for purposes of immunization these errors must be taken into account.

The photométre offers precise accuracy and enables the bacteriologist to estimate vaccines with great rapidity.

The charts plotted show that there is a definite curve and by comparison of the readings for both absorption and diffusion of light an accurate estimation of the strength of the vaccine may be reached.

Photometric estimation of vaccines offers a material aid to the laboratory worker and the photométre of Vernes, Bricq and Yvon appears to be easily adaptable for this purpose.

A BACTERIOLOGIC SURVEY OF COWS IN A CERTIFIED DAIRY

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In as much as few careful studies of individual cows of certified dairies have been recorded, the following experiments and observations seem worth reporting.

In the spring of 1925, the bacterial counts of milk produced by a certified dairy in California suddenly increased, and while there was no outbreak of milk-borne disease among the consumers of the milk, comprising several hospitals, the dairy was placed on probation by the Medical Milk Commission of San Francisco.

Suspicion was at first directed to the bottling machine and later to the cooling chamber, but plate counts made from milk before it entered either machine showed the presence of large numbers of organisms, thus pointing to some difficulty earlier in the process of milk production. Agar plate counts made from individual buckets at milking time resulted finally in the discovery that certain cows were responsible for the high counts. These animals were immediately removed from the barn and sold. A single plate count was also made on each remaining animal, so that, prior to my own connection with the dairy, 25 more were also removed from the main barn and isolated from the milking herd.

July 15, 1925, I was asked to come up to the dairy to see if an epidemic of infectious mastitis was present in the herd.

The sanitary arrangements at the farm were of high character. The main milking barn, accommodating one hundred cows, was a large well lighted, cement structure, with drainage troughs and pipes for carrying away the excreta. It was equipped with running water for washing down the animals before milking and for cleansing the barn after milking. There was a sanitary basin with clean towels for the milkers. The water supply came from a private reservoir situated in the hills above the farm and was fenced off to protect it from human contamination. The dairy also maintained a hospital barn for cows during calving and two isolation barns for animals with infectious diseases.

The milk house was located a short distance from the main barn, and was supplied with live steam for sterilization of equipment. The milk room contained an ammonia cooling plant and a bottling machine, and opened directly into the refrigerator room. All the buckets, cans, and bottles were thoroughly washed in hot running water and placed in a large steam sterilizer for two hours at

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eleven pounds pressure. The vacuum was next turned on to dry the cans and pails and to prevent rusting. The sterilizer was opened shortly before milking time and the cans, pails, and bottles taken into the milk room. The buckets were of the small hole type, and, during milking, the openings were covered with sterile cheese cloth.

All of the milking was done by hand. The gang system was used, each milker being responsible for a certain number of cows. The milkers were dressed in white uniforms.

Two hundred cows were being milked, 175 in the main barn and the 25 which had been removed from the main herd. The feed was carefully selected to stimulate maximum production. Between milking hours, the animals were allowed to graze, and were in the barns only during milking. Owing to climatic conditions, the entire herd was pastured throughout the year. Each animal was examined weekly by a well trained veterinarian.

Milking began at seven o'clock, morning and evening. The herd was milked in two strings, as the barn could accommodate only half at one time. As soon as the cows entered, they were hosed down and the udders and hind quarters wiped with clean sterile cloths. Three cows were milked into each bucket, the milk being taken immediately to the milk house, where it passed over the cooling coils into sterile milk cans or into the bottling machine. The milk was then placed in the refrigerator room pending shipment.

Tests were carried on in the dairy's own laboratory. The laboratory was equipped with an electric hot air sterilizer, a small electric incubator regulated at 37 C., a microscope with oil immersion lens, and a sink with hot running water for cleaning glassware. An adequate supply of petri dishes, pipettes, and cans were available for this work.

A series of agar plate counts was made on the 25 isolated cows on four consecutive days, with a 5th test 18 days later. The main herd was tested weekly for three consecutive weeks. Samples of milk were drawn directly into sterile test tubes from the four quarters. The first few streams were discarded before samples were taken. Whole or middle milk samples from the main herd were not tested unless a high count was obtained from the first milk. First milk samples were used for testing so as to get the cows out of the barn as quickly as possible, with the least possible interference with the work of the milkers. Whole milk samples of the entire herd could not be taken because of the lack of the requisite number of sterile buckets. However, counts of whole and middle milk were made on the isolated group. Each sample was immediately plunged into a bucket of cracked ice and kept there until examinations could be made, usually within two hours. One cc. of a 1-100 dilution was tested and the plates incubated for 48 hours at 37 C.

The milk of those cows suspected of having mastitis was examined for streptococci as follows: samples were collected during the evening milking and allowed to stand in a pail of cracked ice in the refrigerator

room over night. The sediment was then streaked out on blood agar plates and incubated for 24 to 48 hours. Small colonies were stained and examined under the microscope for streptococci.

RESULTS

A white staphylococcus was found in the milk of each of these 25 cows, nine being hemolytic strains. Hemolytic streptococci were not isolated from any of the animals, although two showed nonhemolytic strains, as follows:

One cow had had a severe hemorrhage of the udder, probably traumatic in origin. A long-chained, nonhemolytic streptococcus was isolated from the injured quarter.

One cow (3), with a previous history of mastitis, was also found to have a nonhemolytic streptococcus in one quarter. This quarter was practically dry, giving a very small amount of secretion on pressure.

Table 1 shows the bacterial counts of the first, whole, and middle milk of the isolated herd. The average bacterial count for first milk was 9550, for whole milk 3804, and for middle milk 3860. These results show that the number of organisms in the first streams of milk is about three times greater than that of middle milk or whole milk. Furthermore, nine counts of first milk were above 10,000, while only three of whole milk were above that number. Of these three, two were of animals also showing a high count in first milk.

Three series of tests on the middle milk of these animals showed four with a count above 10,000 in the first series, and two each in the second and third series. All but one of these high counts came from animals showing a previous high count in either first or middle milk. Thus, four cows (19, 20, 21 and 22 in table 1), were found to be frequent high counters. However, one of these animals (20) showed no physical signs of mastitis.

These results showed that most of these cows were not harboring large numbers of bacteria in their udders, while a thorough examination by a well-trained veterinarian showed few with clinical symptoms of mastitis. However, six were removed from the herd for the following reasons:

Three (3, 19 and 21) showed clinical symptoms of mastitis and gave one or more counts above 10,000.

One (10) had one leaky quarter and was isolated until the opening in the quarter could be closed.

One (20) showed no mastitic condition of the udder but was removed from the herd because of several high counts.

One (22) was suspected of having an infected udder before this work was commenced because of frequent high counts, so she was isolated until dry and sent to the butcher.

This work showed that although no true epidemic of mastitis was present in the herd, some of the animals were affected, thus giving rise to an increase in the numbers of bacteria in the mixed milk.

TABLE 1

BACTERIAL COUNTS OF MILK FROM ISOLATED HERD

(Counts per Cc. Based on Dilution of 1–100)

Number of Cow	First Milk July 25	Whole Milk July 26	Middle Milk July 27	Middle Milk July 28	Middle Milk Aug. 15
1	1,600	2,400	600	1,200	400
2	13,100	600	2,000	3,500	3,900
3*	37,900	1,300	2,300	1,600	1,100
4	200	Less than 100	300	1,500	1,100
5	500	1,800	10,500	1,100	4,500
6	4,600	1,700	700	1,100	2,200
7	3,800	300	8,900	2,200	600
8	10,100	400	500	1,300	3,900
9	1,700	5,900	1,700	2,900	2,100
10**	Not countable	9,500	4,500	800	1,600
11	8,100	800	500	.....	.....
12	7,200	100	3,500	1,000	4,800
13	7,700	2,500	1,600	1,100	3,300
14*	2,500	Less than 100	.....	1,700	400
15	23,100	1,200	1,300	2,400	900
16	15,200	3,600	7,000	1,500	4,900
17	3,500	2,500	1,900	200	Dry
18	5,000	600	3,600	1,800	2,300
19*	17,400	1,200	54,100	11,700	14,000
20	49,000	14,100	3,200	300	10,200
21*	3,900	35,500	22,700	15,300	2,000
22	12,200	Not countable	11,500	800	Dry
23	6,200	500	2,700	1,300	2,000
24*	900	1,800	500	1,100	2,700
25	4,700	3,000	900	700	2,400

* Symptoms of garget.
** Leaky quarter.

Three series of plate counts were also made on the 175 cows in the milking barn at weekly intervals.

In the first series of tests, eighteen gave bacterial counts above 10,000, 14 in the second series, and only 9 in the third. The highest count in the first was 27,900, in the second 40,000, and in the third 34,000. Little importance can be attached to the high counts of the first series, ten of which were made during the first few days while the laboratory work was being organized. It soon became evident that the water blanks prepared from tap water were incompletely sterilized.

Spreaders were frequent, even in the controls, and two sterilizations a day apart were found necessary to destroy the spore forms. After the installation of a better autoclave and double sterilization of the blanks, tests were again made on those animals previously showing a high count. These counts, in every case were low, ranging from less than 100 to 6400 organisms per cc. Further tests were controlled both with agar and water to insure accuracy of results. These counts were all well within the limits of prevailing standards for certified milk.

Table 2 summarizes the results of these three series of tests. 3% of the total number of samples were less than 100 per cc., while 66% gave counts below 3000. Moreover, 89% showed counts less

TABLE 2

BACTERIAL COUNTS OF MILK FROM MAIN HERD

(Counts per Cc. Based on Dilution of 1-100)

Bacterial Counts	1st Series	2nd Series	3rd Series	Total Number Counts	Approxmate per Cent of Counts
Less than 100	5	4	6	15	3.0
100-500	26	19	46	91	18.0
500-1,000	12	26	32	70	14.0
1,000-2,000	36	40	27	103	20.5
2,000-3,000	19	19	16	54	10.6
3,000-4,000	16	7	9	32	6.3
4,000-5,000	9	6	10	25	5.0
5,000-6,000	10	7	5	22	4.3
6,000-7,000	8	2	3	13	2.6
7,000-8,000	3	4	3	10	2.0
8,000-9,000	6	1	4	11	2.2
9,000-10,000	2	4	2	8	1.6
10,000-15,000	9	7	4	20	4.0
15,000-20,000	5	1	2	8	1.6
20,000-30,000	6	3	2	11	2.2
30,000-40,000	0	1	1	2	0.4
40,000	1	2	0	3	0.6
Total	..	..	..	508	98.9

than 10,000, the maximum number for certified milk. 8.6% of the total number were over 10,000, while 20.47% of counts, ranged from 1000 to 2000 organisms per cc.

The two counts ranging from 30,000 to 40,000 in the second series of counts were from the same animal. The counts of first milk of this animal ranged from 15,700 to 34,800, while those of whole milk were 5,400 and 3,800 respectively. One quarter was almost dry and gave evidence of a chronic mastitis. She was removed from the barn because of this condition.

Two other animals were also taken from the main barn. The first, although showing no clinical symptoms of mastitis, gave an average bacterial count of 14,650. The second animal had a previous history

of mastitis, and tests of her milk showed her frequently to have large numbers of organisms in her udder. A whole milk count was 18,900.

No explanation can be given for the two counts of 40,000 in the second series of tests, as neither of these animals was near the dry period, nor did they show any involvement of their udders. Other tests on their milk gave very low counts.

The count of 40,000 in the first series was from an animal nearing the end of her lactation period. Several other animals, previous to drying, also gave high counts, although some showed very low counts. There is a general impression among dairymen that the number of bacteria in the udder of a cow tends to increase toward the end of her lactation period. These results support this in part, as 5 of 11 cows showed fairly large numbers.

TABLE 3

PLATE COUNTS OF MILK FROM ANIMALS NEARING END OF LACTATION PERIOD

Number of Cow	1st Count	2nd Count
26	40,300	22,200*
27	600	700
28	100	2,200
29	4,500	700
30	12,700	11,200
31	10,400	11,400**
32	5,700	400
33	11,700	19,000
34	11,300	11,600
35	1,000	1,400
36	1,100	4,200

* Third count, 21,000.
** Third count, 10,500.

DISCUSSION

The number of organisms normally present in the udders of healthy cows is not large. Some observors have reported that freshly drawn cow's milk has some germicidal action. Thus, Heinemann and Glenn,[1] found that fresh cow's milk, when inoculated with pure cultures and incubated at room temperature showed an inhibitory action to the growth of the organisms. This action was found to decrease at 37 C. Heating to 75 or 100 C. destroys it, while 56 C. does not wholly destroy it. These investigators believe that agglutinins may be partly responsible for this action. Chambers,[2] and Hanssen [3] were able to verify this property, the latter worker finding it present during the first four hours after the milk is drawn at a temperature of 37 C. and for longer periods

[1] J. Infect. Dis., 1908, 5, p. 534.
[2] J. Bact., 1920, 5, p. 527.
[3] Brit. J. Exper. Path., 1924, 5, p. 271.

if the tests are carried on at room temperature. Hanssen attributes it to the oxidizing enzymes in the milk. Sherman and Curran [4] inoculated fresh milk with cultures of Streptococcus lactis after they had passed the lag period of growth and were in the period of rapid growth, and found a slight but definite inhibitory effect upon the growth of the organisms. This may be an important controlling factor in the numbers of bacteria normally present in milk as delivered.

Other workers, testing individual cows, have found that the average count of normal animals is low. Hastings and Hoffman [5] found that the average count of healthy animals is not over 1000 organisms per cc. They emphasize the desirability of selecting cows for milk production on low bacterial numbers in their udders. Evans [6] examined 192 samples from 161 cows and found that in 32 (16%), bacteria were not multiplying in numbers worth considering. Davis [7] examined 40 samples of certified milk and found numbers ranging from 2000 to 15,000 per cc., while 46 pasteurized samples gave counts of 10,000 to 45,000. Brown and Orcutt,[8] after a series of examinations, observed certain animals producing milk practically free from bacteria and noted the constancy of the flora. Other animals, however, gave high counts. They too emphasized the need of selecting cows of low bacterial count for milk production.

Several English investigators, Freear, Mattick, and Williams,[9] examining samples of certified milk 24 hours old, found that 12 (6%) of the total number (201) gave counts above 10,000, while samples kept for 30 hours showed 18.7% above 10,000. These counts were made on samples not kept on ice while being shipped to the laboratory.

Frost and Bachmann [10] found bacterial counts of milk from three certified dairies ranging from 3000 to 4000 per cc.

The presence of large numbers of organisms in the udders of cows may not necessarily point to an infection of the organ. Hastings and Hoffman [5] found two cows in one herd producing milk with constant large numbers of organisms. One animal had a leaky quarter, but no marked difference was observed between the leaking and nonleaking quarters. The other animal was normal with no physical symptoms of mastitis, but gave an average count of 33,800.

[4] Proc. Soc. Exper. Biol. & Med., 1924, 22, p. 15.
[5] Univ. Wisconsin, Agric. Exper. Sta., Bull. 6, 1909, p. 189.
[6] J. Infect. Dis., 1916, 18, p. 437.
[7] Ibid., 1918, 23, p. 559.
[8] J. Exper. Med., 1920, 31, p. 49.
[9] J. Hyg., 1921, 20, p. 125; 1921, 20, p. 371.
[10] Am. J. Pub. Health, 1923, 13, p. 300.

It is generally believed that the streptococcus group is the principal cause of mastitis, although other organisms have been associated with this condition. The non-hemolytic streptococcus isolated from these mastitic animals was not tested to differentiate it from the normal udder strains. The cause, therefore, of these few cases, is not known.

Since this bacteriological examination showed no serious epidemic of mastitis in the herd, and after those animals showing either clinical symptoms or high bacterial counts were removed from the milking herd, the dairy was again certified.

CONCLUSION

Bacteriological investigation of individual cows in certified dairies is an important aid in the production of milk with low bacterial content.

THE ACTIVITY OF AN ANTICOLON BACTERIOPHAGE IN SYNTHETIC MEDIUM

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In an attempt to obtain the lytic principle of d'Herelle in a fluid fit for intravenous use, a study has been made of various synthetic media and the development of the bacteriophage in them.

A survey of the voluminous literature of the bacteriophage reveals that in only a few instances has a non-protein vehicle been used in growing the bacteriophage.

D'Herelle [1] states that the true culture medium of the bacteriophage is the living bacterium and that the nature of the fluid in which the bacteria are suspended is without direct influence upon the culture of the bacteriophage, provided only that the bacteria remain living in it throughout a sufficient period of time and that the medium be alkaline in reaction. D'Herelle gives the formula for a synthetic medium and states that serial culture of the antishiga bacteriophage can be accomplished in alkaline salt solution and in chemically pure water containing 25 mg. of soda per liter.

Lepper,[2] using a modified Ayers' synthetic medium, noted the growth curve of B. coli and made serial passages of an anticolon bacteriophage, but reported that a bacteriophage so passed decreased in activity and could not be restored to its original titer by passage in peptone water. The experiments reported in this paper do not confirm these results.

Pozerski [3] devised a protein free medium and produced in it an active filtrate against B. coli which, he states, is practically free of bouillon. However, the medium is not so simple as that of Ayers' used in this study, and he gives no conclusive proofs that the filtrate is protein free.

Synthetic mediums were prepared as described by Uschinsky, Fränkel, d'Herelle and Ayers.[4] The mediums were inoculated with B. typhosus, B. dysenteriae, and B. coli as bacteriophage strains for these bacilli were available. In some instances several combinations of sodium and potassium salts were tried, as the text books did not always state the exact chemical composition of the substances used. The reaction was adjusted to P_H 7.8 to 8.2 in all mediums. B. typhosus and B. dysenteriae grew poorly in all mediums and in only the two latter was the growth of B. coli sufficiently luxuriant for the purpose of the experiment.

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[1] The Bacteriophage, 1922, p. 42.
[2] Brit. J. Exper. Path., 1924, 5, p. 40.
[3] Compt. rend. Soc. de biol., 1925, 93, p. 1285.
[4] J. Bact., 1918, 3, p. 433.

The medium described by Ayers was selected and slightly modified as follows:

Sodium ammonium phosphate	0.4 gm.
Acid sodium phosphate	0.2 gm.
Dextrose	1.0 gm.
Distilled water	100.0 cc.

The dextrose was added as a 20% sterile solution to the autoclaved solution and it was then tubed aseptically with a little ground marble in the bottom of each tube. This medium has a distinct advantage over the others in that it does not contain any substance which gives a biuret test so that the end products of lysis could be tested for the presence of proteins or their derivatives in quantities sufficient to give a positive test.

With these favorable conditions an experiment was undertaken to determine the effect on the bacteriophage of a series of passages in this medium and to study the active filtrates as to their safety for intravenous use.

A strain of B. coli was selected which grew readily in this medium and which has shown no evidence that it is a lysogenic strain throughout a period of two months. The lytic filtrate used was obtained from the urine of a typhoid patient and has a wide activity against typhoid, dysentery and colon strains. All titrations were made according to the serial dilution method of Werthemann,[5] the last tube remaining clear when the control first clouds being taken as the titer number. At the beginning of the experiment, the titer of the bacteriophage against B. coli was 10^3. Tested against a stock typhoid bacillus this same filtrate before passage in synthetic medium had a titer of 10^4.

Serial passage was made using the drop method of d'Herelle. Thus a drop of 24 hour culture of B. coli in the synthetic medium was put into each of several tubes, each containing 8 cc. of medium. The bacteriophage was added in increasing amounts, 2, 10 and 30 drops. Usually two tubes of each amount were set up. Agar slants (1%) were inoculated after two hours incubation and after eighteen hours just before filtering. The cloudy and clear tubes were filtered separately through filters which were used only in this experiment.

There was evidence of lytic action with each passage both in fluid and solid media. The two hour slants showed ragged fringes of growth and large plaques, from 3 to 5 mm. in diameter. Some of the tubes in every passage were clear after 18 hours incubation, usually all but

[5] Archiv. f. Hyg., 1922, 91, p. 255.

one or two. Some of the tubes remained permanently clear thus differing from broth cultures where secondary cultures always developed early not only with this bacteriophage strain but also with any anticolon strains so far encountered by the author. In the clear tubes the P_H value was from 7.4 to 7.8 while the filtrate from the cloudy tubes was distinctly acid. After the first passage the biuret reactions on the filtrates were negative save occasionally in filtrates from the very cloudy tubes.

After ten serial passages, the last filtrate was titrated. Against B. coli in broth it gave a titer of 10^{-7}. Against B. typhosus the titer remained 10^{-4}. In performing titrations using the synthetic medium a small drop of eighteen hour culture must be used instead of the standard loopful and as the growth lag is more pronounced in this medium comparison must not be made with the control until between twelve and eighteen hours. Against B. coli the titer number in synthetic medium varies from 10^{-10} to 10^{-12}. Comparing these titrations with the earlier ones a definite enhancement against B. coli is noted, from 10^{-3} to 10^{-7} in broth and no loss of activity against B. typhosus. The titer number in synthetic mediums is higher because the growth lag is greater in this medium and there is little tendency for secondary cultures to develop. It therefore gives a better idea of the true virulence of a given bacteriophage strain and is comparable to titrations of antidysentery (Shiga) and antityphoid filtrates in broth.

It seemed strange that lysis of the millions of organisms introduced by drop inoculation should produce no substance giving the biuret reaction; so the filtrates were tested to see if they would produce anaphylaxis in animals. Three guinea-pigs were injected intraperitoneally with 5 cc. of the active filtrate. Reinjections were given intracardially, 2, 19, and 21 days later, using two or three cc. of active filtrate for each guinea-pig. None of the animals showed any sign of anaphylaxis. Others were injected intraperitoneally and subcutaneously with reinjections ten days later into the same areas, with similar negative results.

SUMMARY

An anticolon bacteriophage was transmitted by serial passage in a nonprotein medium.

Enhancement of virulence toward the organism used occurred during the passage.

Virulence toward a different organism was not decreased.

Titrations of anticolon filtrates were more satisfactory in synthetic medium than in broth.

The active filtrates did not give the biuret reaction and did not produce anaphylaxis in guinea-pigs.

THE ISOLATION OF SUBSTANCES WITH IMMUNE PROPERTIES

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The amount of protein associated with one hemolytic unit in an amboceptor serum is ordinarily not less than 0.04 mg. This has been reduced by electro-dialytic fractionation [1] to 0.002 mg., and by fractional adsorption [2] to 0.000,125 mg. The best fractionations were obtained with amboceptor serums whose hemolysin content was strongly adsorbed by homologous erythrocytes. This relation has stimulated two lines of effort, the one toward the production of more highly adsorbable or avid serums, and the other toward an explanation. As a result, an hemolysin has been prepared, from an especially avid serum, which contains, approximately, but 0.000,044 mg. of protein per hemolytic unit, and, further, a study of the hemolysin-erythrocyte equilibrium has indicated the nature of antibody structure.

THE QUANTITATIVE MEASURE OF AVIDITY

There are marked differences in the avidity or adsorption affinity of antibodies for antigens and of antigens for their anchoring tissues, and the titer or antibody content of an immune serum is not a complete measure of its therapeutic usefulness.

The quantitative measure of the avidity of an antigen for an antibody preparation requires first a determination of the characteristic neutralization or adsorption equilibrium. This equilibrium is found to be expressed, approximately, for the hemolysin-erythrocyte system, by the equation: [2]

$$\frac{A}{C} = K \cdot \frac{H}{S} \qquad (1)$$

where A and H are the numbers of units of hemolysin adsorbed and unadsorbed respectively when C units of erythrocytes are added to one cc. of hemologous amboceptor serum. K is a constant, and S is

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[1] Locke and Hirsch: J. Infect. Dis., 1924, 35, p. 519.
[2] Ibid., 1925, 37, p. 449.

the number of units of an undetermined, hemolysin binding substance in the serum.

The quantity $\frac{A}{C}$ represents the amount of hemolysin which can be extracted from a given serum by a given amount of erythrocytes, per unit of those erythrocytes. It becomes increased in value as C, the amount of added cells, is made smaller, and reaches a maximum as C approaches zero. But as C approaches zero, H, the amount of hemolysin left unadsorbed in the serum, approaches a value equal to the original titer of the serum, i. e.:

$$\frac{A}{C}\ (\text{maximum}) = \frac{H\ (\text{maximum})}{\frac{S}{K}} \qquad (2)$$

which states that:

The avidity, or maximum adsorbability of the serum hemolysin upon homologous erythrocytes. $\propto$ $\frac{\text{The titer of the serum.}}{\text{The binding-substance content of the serum.}}$ $\propto$ $\frac{1}{\text{The binding-substance content of the serum, per hemolytic unit.}}$

The avidities of several amboceptor serums have been measured and collected in table 1.[3, 4] The measurement has been carried out simply

TABLE 1

THE COMPARATIVE AVIDITIES OF ANTISHEEP HEMOLYSINS PREPARED IN DIFFERENT WAYS *

Hemolysin		Titer, Units per Cc.	$\frac{S}{K}$	Avidities for Sheep Cells	Avidities for Guinea-Pig Kidney Cells
"Isophile," produced by the injection of sheep corpuscles into a guinea-pig (Singer)[3]	A	125	10.5	11	>0
	B	50	3.0	11	>0
Produced by long continued intraperitoneal injections of sheep cells into a rabbit	C	700	10.0	70	.....
	D	500	1.25	400	.....
Produced by two intravenous injections of sheep cells into a rabbit, spaced according to Coca.[4]	E	1,500	2.25	666	4,300
Produced by three intraperitoneal injections of sheep cells into a rabbit	F	1,700	0.61	2,800	.....
	G	1,000	0.44	2,300	.....
Produced by three intravenous injections of sheep cells into a rabbit	H	8,250	3.13	2,650	.....
	I	1,700	0.51	3,350	.....
"Purified," from serum I by selective adsorption and iso-electric precipitation	J	1,700	0.28	6,100	.....
Produced by two intravenous injections of sheep cells into a rabbit (massive)	K	5,000	0.86	5,800	50,000
Produced by one intravenous injection of sheep cells into a rabbit	L	1,300	0.08	16,250	43,350
	M	1,500	.047	32,000	107,000
	N	1,250	.008	156,000	.....
"Heterophile," produced by two subcutaneous and one intraperitoneal injection of guinea-pig kidney emulsion	O	4,000	1.2	3,350	40,000
"Natural," normal serums, human.....	P	18	.025	720	5 000
hog.....	Q	25	.09	275	$<\infty$
rabbit.....	R	7	<.003	>2,500	.....

* Unless otherwise stated, an interval of one or two days elapsed between succeeding injections, and the animal was bled from the heart ten days after the last injection. The computed values are probably correct within ± 16%.

[3] Ztschr. f. Immunitätsf. u. exper. Therap., O., 1922, 35, p. 191.

[4] J. Infect. Dis., 1915, 17, p. 361.

by determining the amount of hemolysin which is adsorbed by one, two, and three units [2] of cells, respectively, from one cc. of the hemolysin serum, and substituting the values so determined in equation (1). The value of $\frac{S}{K}$ is thus measured, and the quotient of the serum titer by this value (equation 2) gives the avidity. This seems to depend upon the number, spacing, and method of making the antigen injections, since these all affect the titer of the amboceptor serum obtained and the amount of binding substance dispersed in it.

A similar conclusion has been reached independently by Barikin and Friese [5] who have found that the avidity of immune serums produced by a great number of antigen injections is much less than that of the serums produced by a smaller number. Their conclusion is based upon the fact that the rate of adsorption by erythrocytes of the hemolysin from the former type of serum is much slower than from the latter type.

THE RELATION OF AVIDITY TO SPECIFICITY

The specificity of rabbit anti-sheep hemolysins is greatly increased, according to Taniguchi,[6] by repeated injections of antigen, a course which greatly diminishes their avidity. Guinea-pig anti-sheep hemolysin, which has been selected by a great number of investigators as a model of isophile or perfectly specific hemolysin, combines slowly with added erythrocytes. Maximum adsorption is reached still more slowly by toxins and antitoxins, whose exceedingly low avidities are no doubt due to the prolonged course of antitoxin immunization against repeated and gradually increased doses of toxin.

The therapeutic properties of the antitoxins have been determined with serums of exceedingly poor avidity and high specificity. A new type of clinical experiment should be undertaken now that antitoxins may be purified and separated from a portion of their binding substance by isoelectric fractionation. It is conceivable that antibody preparations more highly avid and less specific, corresponding more closely, in fact, to the antibodies naturally present in the blood, may be quicker and more versatile in their neutralization of bacterial toxins than the immune serums now available.

The correlation of low avidity with high specificity is more than a coincidence. Iron filings are not attracted to a magnetic surface unless there is sufficient magnetic attractive energy exerted to overcome their inertia. Nor can the act of adsorption between an antibody and an antigen occur unless there pre-exists a critical amount of potential energy capable of accomplishing the adsorption. The amount of this energy is related to the avidity of the antibodies, and highly avid prepara-

[5] Ztschr. f. Immunitätsf. u. exper. Therap., O., 1925, 45, p. 191.
[6] J. Path. & Bact., 1921, 24, pp. 217, 241, 456.

tions can be adsorbed by a much greater variety of antigens and to a much greater extent than the less avid. They therefore appear to be less specific.

The property of specificity is, then, linked directly with the amount of binding substance dispersed in the serum, i. e.:

The degree of specificity of a serum. $\propto f \frac{1}{\text{Avidity}} \propto f$ The binding substance content of the serum, per hemolytic unit.

But the fundamental, specific character of an antibody, which determines how much potential energy represents the critical amount capable of accomplishing adsorption, is surely determined by the structure of the antibody itself. It seems pertinent to inquire if specificity may not be influenced by the character, as well as the amount of the dispersed binding substance, and if this binding substance may not be an integral part of the hemolysin structure.

THE RELATION OF THE AVIDITY TO THE PROTEIN CONTENT OF PURIFIED HEMOLYSIN

It has not as yet seemed advisable to depart much from the general method of purification of amboceptor serums reported in a previous paper.[2] This method has been used for the fractionation of a large number of hemolysin preparations of different origin, and the data relative to the titer and avidity of the original serum, and the yield and purity of the recovered hemolysin are reported in table 2. The nitrogen determinations were made by the method described by Main and Locke.[7]

The results demonstrate a direct relation between the avidity of the original serum and the purity of the recovered preparations. They seem to indicate that the hemolysin from a poorly avid serum contains more protein than the hemolysin of a highly avid serum. This relation is not dependent upon the volume of cells used in the original adsorption and is therefore not due to differences in the unspecific adsorption of serum proteins excepting as these may be incorporated in the structure of the adsorbed hemolysin. (The binding substance may tend to hold a limited amount of unspecific protein to the hemolysin in a way which will be discussed later.)

It has thus far been possible to carry the purification of a hemolysin preparation only to a characteristic optimum value, when the amount of protein associated with the hemolytic unit is at a minimum. The best preparation, from the most highly avid serum which we have been

[7] J. Biol. Chem., 1925, 64, p. 75.

TABLE 2

RELATIONS OF THE PURITIES OF THE BEST HEMOLYSIN FRACTIONS PREPARED FROM AMBOCEPTOR SERUMS BY COMPARABLE PROCEDURES TO THE ORIGINAL AVIDITIES OF THOSE SERUMS

Production by	Amboceptor Serum		Units of Cells Added	Units of Hemolysin Adsorbed per Unit of Added Cells	% Adsorbed Hemolysin Recovered	% Total Hemolysin Recovered	Mg. Protein[7] Associated with Each Unit in the Best Fraction of Recovered Hemolysin
	Hemolytic Titer	Avidity					
Single injection, intravenous, sheep cells	1,250	156,000	2	625	16	16	0.000,044
Three injections, intravenous, sheep cells	2,500	35,700	4	625	19	19	0.000,156
Single injection, intravenous, sheep cells	1,300	16,250	2	625	10	10	0.000,15
Two injections, intravenous, sheep cells	5,000	5,800	2	1,750	11	8	0.000,19
Three injections of guinea-pig kidney cells	4,000	3,350	2	1,250	9	6	0.000,28
Single injection, sheep cells, delayed bleeding	2,500	2,500	4	500	20	15	0.000,28 (Fractionated to an avidity of 3,500)
Three injections, intravenous, sheep cells	1,700	3,350	4	380	65	59	0.000,22 (Fractionated to an avidity of 6,100)
Three injections, intravenous, sheep cells	8,250	2,650	2	1,625	12	5	0.000,29
Three injections, intravenous, sheep cells, second bleeding	5,000	2,500	1	1,700	12	4	0.000,33
Pooled, 1 and 3 injections	1,450	700	4	240	20	13	0.001,14
Prolonged immunization, intraperitoneal	700	70	4	50	100	29	0.002,5 (Not completely fractionated)

able to secure, contains 0.000,000,007 grams of nitrogen per hemolytic unit, and sensitizes guinea-pigs to fatal anaphylactic shock with rabbit serum, as noted in table 3.

The values of table 2 are again compared in figure 1. The abscissae record the amount of protein and the ordinates the amount of binding substance associated with the hemolytic unit. (This latter value is the reciprocal of the avidity, as noted in equation 2). The

TABLE 3

THE CAPACITY OF HIGHLY PURIFIED IMMUNE HEMOLYSINS TO SENSITIZE GUINEA-PIGS TOWARD ANAPHYLACTIC SHOCK AGAINST RABBIT GLOBULIN

Source of Substance for Initial Injection	Sensitization Dose, Intracardial		Mg. of Protein per Hemolytic Unit	Shock Dose, Intracardial, Cc. Normal Rabbit Serum 1:10	Anaphylactic Response
	Hemolytic Units	Mg. of Protein			
Normal serum (control)	.....	0.044*	.....	1.5	Negative
	.....	.044*	.....	1.5	Slight
Hemolysin preparation	800	.125	0.000,156	1.5	Fatal
Hemolysin preparation	1,650	.063	.000,044	2.0	Marked
	1,650	.063	.000,044	2.0	Fatal
(Control)	.....	.....	.....	2.0	Negative

* Approximately 0.03 mg. of this amount is rabbit globulin.

TABLE 4

THE COADSORPTION OF HEMOLYSIN AND OF HEMOLYSIN BINDING SUBSTANCE FROM AN AMBOCEPTOR SERUM BY ADDED ERYTHROCTES

Units of Erythrocytes Added	Units of Hemolysin in Supernatant (Unadsorbed)	Binding Substance Content of Supernatant, ÷ K	Units of Hemolysin Adsorbed	Units of Binding Substance Adsorbed, ÷ K
0	640*	0.34*	0	0
0.2	400	0.30	240	0.04
.4	300	0.27	340	.07
8	200	0.20	440	.14
1.2	140	0.18	500	.16

* The values are probably accurate within ± 15%.

direct linearity of the relation between these two properties is remarkable. It indicates that:

The amount of protein, per hemolytic unit, in a purified hemolysin preparation.	$\propto$	A constantly associated amount of protein.	plus	The amount of protein contributed by the binding substance.

And it implies that when the hemolysin of an amboceptor serum is adsorbed by added erythrocytes, a certain proportion of the binding substance content of the serum becomes coadsorbed with it.

This has been confirmed by the results reported in table 4. Identical amounts of an amboceptor serum were treated with progressively increased amounts of sheep cells and the binding substance value, $\frac{S}{K}$, before and after the adsorption measured as before. The values demonstrate that there is a drop in the binding substance content of the serum after adsorption and that the amount of the drop is roughly proportional to the amount of hemolysin adsorbed.

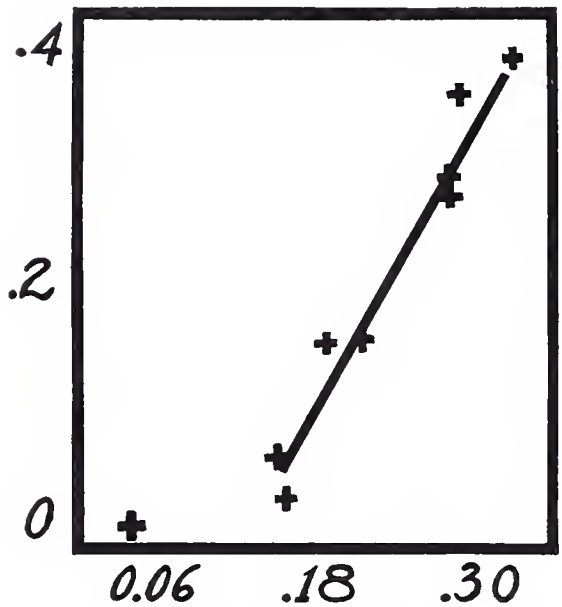

Fig. 1.—The relation between the content of protein and of binding substance in purified hemolysin. The abscissas represent mg. of protein, and the ordinates units of $\frac{S}{K}$ (binding-substance), per thousand units of the purified hemolysin preparation.

The relative proportions adsorbed are compared against in figure 2. It may here be seen that the hemolysin which is first adsorbed carries with it less of the binding substance than that which is last adsorbed; in other words, (equation 2), the most highly avid fractions of the hemolysin in the serum are adsorbed first and the least avid last.

THE RELATION OF THE AVIDITY TO THE SIZE OF THE PURIFIED HEMOLYSIN AGGREGATE

It is possible, on the basis of the data which has been presented, to obtain something of an idea of the relative size of the hemolysin aggregate.

The volume of protein associated with one hemolytic unit per erythrocyte of that unit, multiplied by the avidity of the hemolysin preparation, gives the volume of hemolytic protein adsorbed by an erythrocyte at saturation. This value, divided by the surface area of the erythrocyte, gives the thickness of the adsorbed layer. The thickness of the layer for the preparations listed in table 2 is computed and recorded in table 5. The method of calculation is illustrated in the following example: The volume of protein associated with one hemolytic unit of the first preparation, per erythrocyte of the defined unit, is:

$$\frac{0.000,000,044}{700,000,000} \cdot 10^{21} \text{ or } 60 \times 10^3 \ \overline{\mu\mu}^3.$$

TABLE 5

THE COMPUTED SIZE OF THE PURIFIED HEMOLYSIN AGGREGATES FROM AMBOCEPTOR SERUMS OF DIFFERENT AVIDITIES

Mg. Protein Associated with Each Unit in the Purified Hemolysin	Approximate Avidity of the Purified Hemolysin	Probable Average Diameter of the Hemolysin Aggregate (Approximate) $\mu\mu$	Approximate Distribution of Protein in the Aggregate, Mgs. per 1,000 Units		
			Binding Substance	"Antibody" Protein	Associated Protein
0.000,044	156,000	300	0.003	0.04	....
0.000,15	16,250	100	0.03	0.04	0.08
0.000,19	6,300	60	0.08	0.04	0.07
0.000,22	6,100	60	0.08	0.04	0.10
0.000,28	3,500	50	0.15	0.04	0.09
0.000,33	2,500	40	0.20	0.04	0.09
0.001,14	700	40	0.70	0.04	0.40

This may be compared with the volume of a molecule of globulin, which is approximately $60 \ \overline{\mu\mu}^3$. The estimated surface of one erythrocyte is about $3 \times 10^7 \ \overline{\mu\mu}^2$, containing approximately 10^6—10^7 molecules of lipoprotein. This surface can adsorb from this preparation 156,000 times $60,000 \ \overline{\mu\mu}^3$ of hemolysin, which, significantly, corresponds to about 10^8 molecules of protein and would cause a surface coating $\frac{10^{10} \ \overline{\mu\mu}^2}{3 \times 10^7 \ \overline{\mu\mu}^2}$ or $300 \ \overline{\mu\mu}$ thick, eighty times as thick as a monomolecular coating of globulin, indicating that the hemolysin aggregates from this preparation have an average diameter which is only a little more than ten times less than that of the sheep erythrocyte, although its volume is about a thousand times smaller. The aggregates from an ordinary amboceptor serum are considerably smaller, the computed diameter being roughly proportional to the avidity of the preparation from which it is adsorbed.

THE STRUCTURE OF HEMOLYSIN

The binding substance of an amboceptor serum would seem, a priori, to be derived, for the most part, from the injected antigen and to be of a complex lipoid and lipoproteid structure.[2] The amount of protein associated with one unit of binding substance may be estimated from the data in figure 1. Extrapolation of the graph to zero concentration of binding-substance gives the amount of protein, other than binding-substance, contained in these preparations. This value, subtracted from the value for the total protein at any point on the graph gives the

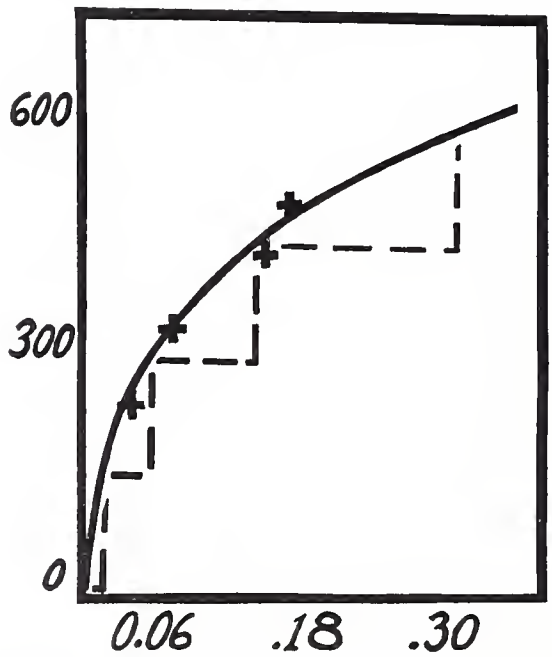

Fig. 2.—The relation of the amount of hemolysin adsorbed by homologous erythrocytes to the amount of coadsorbed binding substance. The ordinates represent the number of units of hemolysin and the abscissas the number of units of binding substance adsorbed and coadsorbed, respectively, when progressively increased quantities of homologous erythrocytes are added to an amboceptor serum.

amount of the protein in an hemolysin preparation which is incorporated in the binding substance. One unit of binding substance, corresponding to 0.025 cc. of sheep cells, contributes, apparently, 0.000,5 grams of protein. This is a very probable value, for the total protein contained in this amount of corpuscles is about 0.001 to 0.0015 grams, all of which is not capable of binding hemolysin.

In the calculated distribution of protein in the antibody aggregates, as recorded in table 5, one can almost see an Ehrlich-like relation, with binding substance lipoid holding the binding substance and "antibody"

proteins together, and, with some residual affinity, attracting unspecific proteins until a favored particulate dimension is reached.

Hemolysin and a substance capable of binding hemolysin could hardly be present in the same serum without being more or less firmly bound together. Each unit of binding substance is probably bound to its proportionate quota of hemolysin with a degree of firmness that would produce an ultramicroscopic cluster or aggregate.

It is significant that an erythrocyte surface, when sensitized with the proportionate part of two units of hemolysin, is capable of sensitizing another surface although its own contains several thousand unsaturated "valencies," and a true dissociation of antibody from antigen has never been demonstrated.[8] This fact becomes clear if the adsorbed hemolysin is held, not as a film, but as a projecting large cluster, the anterior surface of which can only combine with another erythrocyte surface.

The electrophoretic behavior of agglutinated cells, which has been attributed to the formation of a surface film of antibody or globulin, is just as clearly explained by the assumption of the adsorption of even a single cluster of antibody. This single cluster may contribute a type of dissociation which influences the electrophoretic behavior of the entire adsorption system. (A single electron has been demonstrated to change the electrophoretic behavior of a particle of suspensoid although it could hardly be assumed to form a completely encasing film around the particle.)

The magnitude of the antibody clusters is demonstrated to be such that, while many thousand times smaller than an erythrocyte, they may serve to hold together or agglutinate cell material with the same simplicity that a droplet of glue holds together a group of pith balls.

THE ARTIFICIAL PRODUCTION OF A TRANSITION IN THE CHARACTER OF ANTIBODIES

If hemolysins are constituted of aggregates or clusters of large dimension in which an elementary, naturally occurring protein substance is adsorbed upon nuclei of a binding substance derived from the injected antigen, and if the properties of immune hemolysins of different origins are due to variations in the character and proportion of the binding substances present in the clusters, these properties should be susceptible of artificial change through changes in the proportion and character of the binding substance in the clusters.

The proportion of binding substance may be reduced by fractionation, reducing the amount of protein associated with the hemolytic unit and increasing the avidity of the preparation.

The character of the binding substance may be partly changed by the artificial introduction into the hemolysin clusters of the binding

[8] Huntoon, F. M.: J. Immunol., 1921, 6, p. 117.

substance of an antigen. The most satisfactory material for this experiment is the so-called, natural hemolysin. This hemolysin differs from an immune hemolysin in being less thermostable [9] and less specific in its adsorption tendencies.

Boissevain [10] has reported that a suspension of natural hemolysin upon erythrocytes has the specific characters of an homologous immune serum, and we have found, as reported in table 6, that an artificial dispersion of natural hemolysin upon erythrocyte material is as thermo-

TABLE 6

TRANSITION OF THERMOLABILE TO THERMOSTABLE HEMOLYSIN AS A CONSEQUENCE OF ADSORPTION UPON ERYTHROCYTES FOLLOWED BY DISINTEGRATION OF THE ADSORPTION SEDIMENT

	Total Hemolytic Titer, in Units			
	vs. Sheep Cells		vs. Guinea-Pig Cells	
	Before Heating	After Heating ½ Hour at 62 C.	Before Heating	After Heating ½ Hour at 62 C.
Normal rabbit serum	280*	0	100*	0
Supernatant after adsorption on 0.5% sheep cells	4	0	50	0
Suspension of the ether-extracted, disintegated adsorption sediment	190	190	0	..
Immune hemolysin	5	5	...	..
Heterophile hemolysin	3	3	...	..
Normal rabbit serum	280	0	100	0
	140	...	50	..
Supernatant after adsorption on 0.5% guinea-pig erythrocytes	280	0	30	0
	140	...	6	..
Suspension of the ether-extracted, disintegrated adsorption sediment	35	35	0	..
	37	37	0	..
Normal ox serum	0	...	900	..
Supernatant after adsorption on 0.5% guinea-pig erythrocytes	0	...	800	..
Suspension of the ether-extracted, disintegated adsorption sediment	0	...	0	..
Neutralized, isotonic, acid extract of the sediment	12	...	0	..

* The computed values are probably correct within ± 15%.

stable as an immune or hetorophile hemolysin. It may be noted that the process of disintegration and dispersion causes a transition in the specific character of adsorbed natural guinea-pig hemolysins. This is very suggestive, for the guinea-pig erythrocyte is a partly heterogenetic antigen.[6]

A transition in the thermolability of immune serums during the early stages of immunization was noted in 1914 by Thiele and Embleton [11]

[9] Kolmer, J. A.: J. Immunol., 1919, 4, p. 403.

[10] Compt. rend. Soc. de biol., 1922, 87, pp. 1255, 1257.

[11] Ztschr. f. Immunitätsf. u. exper. Therap., 1914, 20, p. 1.

in their interesting paper, "The Evolution of Antibody." The first transition in the specificity of an antibody was recorded in 1913 by Bail and Rotky.[12] They reported that the antibodies dissociated by heat from an antigen sensitized with normal serum are endowed with the isophile character of immune substances, and proposed a theory of the origin of the immune substances in vivo that had for its fundamental premise an idea that natural antibodies are converted into immune antibodies as a result of the temporary contact of the antibody with the antigen during adsorption. The premise was likewise the keystone of the claim of Ostromuislenski[13] that antibodies can be synthesized from ordinary proteins by inducing the adsorption of the proteins upon the requisite antigen through the action of a catalyst. We have repeated a part of the work of these papers[12, 13] and have reached considerably

TABLE 7

PRESENCE IN THE SERUM FROM POST PARTUM BLOOD AND IN THE SERUM OF LATE PREGNANCY OF A SUBSTANCE CAPABLE OF DISINTEGRATING ERYTHROCYTES SENSITIZED WITH NATURAL HEMOLYSIN

Human Serum	Original Titer in Units per C c.	Supernatant Titer After	
		Short Contact with 0.6% Sheep Erythrocytes	Prolonged Contact with 0.6% Sheep Erythrocytes
Early pregnancy	10	0	0
Late pregnancy	10	4	5
Retroplacental	16	3	6
Hypertension, day before delivery	25	1	22

different conclusions. Bail and Rotky's best results were obtained with cholera vibrio, an organism that is not highly stable when suspended in serum and which tends to become disintegrated and dispersed in a manner which favors the inclusion of bacterial binding substance in the antibody clusters with the resulting changes in thermostability and specificity already discussed.

Their best results in the production of hemolysins were with the sensitization of erythrocytes with serum obtained from post partum blood. We have found that this serum contains a substance which is not ordinarily present in the blood excepting perhaps in the late stages of pregnancy and perhaps in certain pathological conditions, which is partially adsorbed by heterologous erythrocytes and is capable of slowly disintegrating them even at ice-box temperature. The supernatant titer of a natural hemolytic serum is usually the same, or even less, after

[12] Ibid., 1913, 17, pp. 378, 566; 1914, 21, p. 202.
[13] J. Russ. Phys. Chem. Soc., 1915, 47, p. 263.

prolonged contact with an erythrocyte suspension as after a shorter contact. But the supernatant post partum blood serum, as recorded in table 7, not only gains in hemolysin content after the longer contact, as indicated, but there is visible evidence of an actual disintegration of the cells. This action was so much more rapid at 38 as to make titrations very difficult. There was no appreciable bacterial contamination.

THE MECHANISM OF THE FORMATION OF ANTIBODIES IN THE LIVING ORGANISM

It is possible that the mechanism of the production of immune antibodies in the living organism is likewise a mechanism of disintegration of adsorption aggregates of the injected antigen with natural antibodies,

TABLE 8

THE DEGREE OF ADSORPTION OF HEMOLYSIN FROM HETEROPHILE AND ISOPHILE SERUMS BY SHEEP ERYTHROCYTES AND BY GUINEA-PIG KIDNEY CELLS

	Total Hemolytic Titer with Sheep Erythrocytes			% Adsorbed by	
		After Adsorption on			
Serum	Before Adsorption	1% Sheep Cells	1% Guinea-Pig Kidney Cells	Sheep Cells	Guinea-Pig Kidney Cells
Immune	1,500	1,285	700	14	53
	5,500	3,750	1,000	32	82
	1,500	160	50	89	97
Heterophile	250	28	7	88	97
	4,000	2,900	750	28	81
Normal, hog	100	48	0	52	100
Normal, human	72	16	2	78	97

during which fragments of binding substance from the antigen become included in the antibody clusters.

The content of natural hemolysins in normal blood plasma is variable and is so readily replaced and augmented after frequent bleedings or injections of such substances as colloidal manganese [14] as to suggest an adsorption equilibrium in which the hemolysin free in the plasma represents only a small percentage of a larger amount fixed in the tissues.

Both natural, heterophile, and even immune hemolysins [6, 15] are strongly adsorbed by the so-called heterogenetic tissues, as is indicated in table 8, in which the degree of adsorption of hemolysin by guinea-pig kidney cells is compared with its degree of adsorption by sheep erythrocytes.

[14] Mackie, T. J.: J. Hyg., 1925, 24, p. 176.

[15] Gutfeld, F. v.: Ztschr. f. Immunitätsf. u. exper. Therap., O., 1922, 34, p. 524.

It is significant that the phenomena of anaphylactic shock which follow the too rapid mobilization of antibodies concern these same heterogenetic tissues—the heart, lungs, etc., in the guinea-pig, but principally the tissues in the splanchnic region in the rabbit, whose organs are not markedly heterogenetic. The mobilization of the natural antibodies may perhaps involve only the desorption from heterogenetic tissues of a normal proteinaceous adsorption.

The naturally occurring hemolysins are rapidly adsorbed by injected erythrocytes with the production of a negative phase during which their concentration in the plasma becomes too small to be detected. Adsorption persists until the stroma is saturated and no longer antigenic [16] nor capable of anchoring to the tissues, or, in the excess of antigen, until the animal's supply of normal hemolysins is exhausted. (The duration of the adsorption period is proportional to the size of the animal and to the amount of antigen injected, within certain restriction.) [17] Then, as the result of phagocytosis or of an enzyme action, the unwieldy aggregates are progressively disintegrated [2, 18] and hemolysins again become demonstrable in the plasma. The first liberated hemolysin probably contains very little of the antigenic material and corresponds more closely to the original, natural hemolysin aggregates.[11] Late in the period of progressing immunization the further disintegration of the aggregates probably paves the way to a partial resorption of the antibodies by the heterogenetic tissues, in a condition, however, resulting from some residual inclusion of antigen binding substance in the clusters, which permits of their more rapid mobilization after later injections of antigen.

In conclusion, the writers wish to state that while there has been considerable theoretical elaboration of the fundamental idea of the cluster structure of antibody, this is in no sense offered as an established fact but only as an extremely probable concept. It is hoped that if many of the deductions here presented should ultimately prove to be untenable that they may nevertheless stimulate contemporary investigations into the nature and properties of antibodies.

SUMMARY

The nature of the hemolysin-erythrocyte equilibrium indicates that there is in amboceptor serum a substance, probably derived from the

[16] Iijima, T.: J. Path. & Bact., 1925, 28, p. 397.
[17] Armstrong, R. R.: Proc. Roy. Soc., 1925, B98, p. 525.
[18] Carrel and Ingebrigtsen: J. Exper. Med., 1912, 15, p. 287.

injected antigen, which is capable of binding hemolysin and restraining its adsorption upon added cells. The degree of the characteristic properties of an immune serum, such as specificity, avidity, and thermostability is shown to be dependent upon the amount and character of this binding substance dispersed in the serum. The substance is adsorbed by erythrocytes together with hemolysin. When hemolysin is recovered from adsorption, its protein content is roughly equal to the sum of a constantly associated quantity of protein plus the calculated protein content contributed by the binding substance. The properties of the hemolysin may be partly changed by changing the proportion and character of its binding substance. It is postulated that antibodies are composed of clusters, of relatively large dimension, in which an elementary, naturally-occurring, protein substance is adsorbed upon nuclei of a binding substance derived from the injected antigen, and that they owe their individual properties to the proportion and character of this binding substance. A mechanism is suggested for the production of immune antibodies in the living organism.

THE PRECIPITIN REACTION IN THE IDENTIFICATION OF SCARLATINAL HEMOLYTIC STREPTOCOCCUS INFECTIONS *

FURTHER RESULTS

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The demonstration by Dick and Dick [1] that scarlet fever is due to a hemolytic streptococcus, and the occurrence of many cases of hemolytic streptococcus infections of the throat without rash, both in persons who have had and those who have not had scarlet fever, especially during epidemics of scarlet fever, call for a simple method to differentiate scarlatinal and nonscarlatinal hemolytic streptococcus infections.

In previous reports,[2] I have shown the value in this respect of the precipitin reaction with cleared extracts of nasopharyngeal swabbings or pus from wounds and a scarlatinal antistreptococcus horse serum prepared by repeated injection of scarlatinal hemolytic streptococci. A close agreement was found between a positive precipitin reaction, the clinical and epidemiologic findings, the toxin production, the Dick toxin neutralization test,[3] and the Dick skin reaction.[4]

In this paper I wish to report the important findings noted during a small isolated outbreak of scarlet fever, particularly the comparative value of the precipitin reaction with my old scarlatinal antistreptococcus horse serum and a sample of the concentrated scarlatinal antitoxin kindly furnished by the Dicks to detect scarlatinal hemolytic streptococcus infection.

TECHNIC

The technic was similar to that used in my previous studies. The cleared extracts layered over the undiluted serum and antitoxin gave the best results. If the amount of exudate obtained on the swab from the nasopharynx was unusually large, it was washed off in 4 cc. of sodium chlorid solution, if not so large, in 2 cc. of the solution. After thorough mixing, the suspension was centrifuged at high speed until the supernatant fluid was water-clear or only slightly opalescent.

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[1] J. Am. M. A., 1923, 81, p. 1166.
[2] [a] J. Infect. Dis., 1925, 36, p. 525; [b] J. Am. M. A., 1926, 86, p. 9.
[3] Dick, G. F., and Dick, G. H.: J. Am. M. A., 1925, 84, p. 802.
[4] Ibid., 1924, 82, p. 265.

To check the results with the scarlatinal antistreptococcus serum (Horse 10) and the concentrated scarlatinal antitoxin, normal horse serum and other antistreptococcus serums, namely, poliomyelitis and encephalitis antistreptococcus horse serums and a nonscarlatinal hemolytic antistreptococcus serum, were used as controls in this as in previous studies. Since the results with the use of these serums were negative and in accord with previous findings [2b] they are not considered further in this paper. Because normal horse serums have given uniformly negative results the use of a concentrated solution of the globulin fraction of normal horse serum to control the results with the concentrated scarlatinal antitoxin has seemed superfluous.

RESULTS

The age of the patient, the clinical findings, the occurrence of a rash, the reaction to the Dick skin test, the results of cultures on blood agar plates, the precipitin reaction with the old scarlatinal antistreptococcus horse serum (H10) and the Dick antitoxin in each case, are summarized in the accompanying tabulation. No one in the series had had scarlet fever. The infection in patients 2, 3, 4, 5, 7 and 9 appeared to be due to exposure to patient 1 with a cellulitis following injury, pharyngitis and suppurative otitis media. The rash did not develop in this case. The reaction in the skin test was negative, and the cleared extracts of the discharge from the left ear, of the nasopharyngeal swabbing, and the pus from the right cervical gland, revealed hemolytic streptococci and gave a positive precipitin reaction during his illness. Fifty days later, after the discharge from the ear had ceased, hemolytic streptococci were still present in the nasopharynx but the precipitin reaction had become negative.

In six cases (1, 2, 3, 5, 6 and 7) the clinical findings, including the source of infection, indicated that the illness was due to scarlatinal hemolytic streptococci, and with both serums the precipitin reactions for each patient were positive. Two developed a rash, but in the remaining four the reaction of the skin test was negative and no rash appeared. The cause of the mild febrile reaction in the sister (4) of three of the other patients (1, 2 and 3) remains uncertain. Hemolytic streptococci were not found and the precipitin reaction was negative. One of the four (8) and the maid of the household (4, not studied bacteriologically) were exposed to patient 1. Both developed follicular pharyngitis and tonsillitis about a week before his brothers (2 and 3) developed scarlet fever.

SUMMARY OF CASES

Patient	Age, Years	Clinical Findings	Rash	Reaction to Dick Skin Test	Source of Material	Day of Disease	Cultures, Hemolytic Streptococci	Precipitin Reaction with Scarlatinal Antistreptococcus Serum: Rosenow H10	Precipitin Reaction with Scarlatinal Antistreptococcus Serum: Dick Antitoxin
1	7	Cellulitis right side of neck and head, following injury to right ear; pharyngitis and otitis media; suppurative cervical lymphadenitis, right side. No exposure to scarlet fever	0	0	Discharging ear	10	Many	+	+
						14	Many	+	+
					Nasopharynx	12	Many	+	+
					Pus, cervical gland	16	Many	+	+
							Few	++	++
					Nasopharynx	50	Few	0	0
2*	8	Typical scarlet fever. Drop in temperature following administration of scarlatinal antitoxin. Severe gastro-enteritis 4 days later	+++	?	Nasopharynx	2	Countless	++	++
					Nasopharynx	6	Countless	+	+
					Nasopharynx	44	Few	0	0
3*	4	Typical scarlet fever. Prompt drop in temperature following administration of scarlatinal antitoxin. Severe gastro-enteritis 5 days later followed by pelvic abscess	+	+++	Nasopharynx	1	Countless	++	++
						5	Countless	+	+
					Pus, pelvic abscess	14	Countless	++	?
					Nasopharynx	42	Few	0	0
4*	0.75	Slight fever without throat findings	0	++	Nasopharynx	2	None	0	0
5*	48	Marked angina, fever, headache, nausea and severe aching	0	0	Nasopharynx	2	Countless	++	+
6†	29	Chills, fever, and muscular pains; marked angina	0	0	Nasopharynx	3	Countless	+	++
7‡	51	Headache, chills, nausea, aching, fever, marked angina and follicular tonsillitis	0	0	Nasopharynx	2	Countless	+	++
8*	4	Sore throat, slight fever, follicular pharyngitis	0	++	Nasopharynx	5	Many	0	0
9*	22	Sore throat, moderate aching due to follicular tonsillitis 2 days after exposure	0	0	Nasopharynx	5	Many	0	0
10	56	Severe widespread erysipelas and angina; abscess under left eye	0	?	Nasopharynx	3	Many	0	0
					Palate	3	Many	0	0
					Abscess	2	Many	0	0
11	8	Typical follicular pharyngitis, fever, nausea and vomiting	0	++++	Nasopharynx	2	Countless	0	0
12	26	Typical follicular pharyngitis and tonsillitis	0	+++	Nasopharynx	10	Few	0	0

* Exposed to patient 1.
† Exposed to patients 2 and 3.
‡ Exposed to patients 1 and 4.

About six weeks after the infection in the family began one patient (9) developed symptoms within three days after working as a maid in the household of three of these patients (1, 2 and 3), while they were still carriers of the hemolytic streptococcus. The remaining two (11 and 12) were brother and sister from a distant city. In three of these patients (9, 11 and 12) the reaction to the skin test was positive, and a rash should have developed if the infection were due to scarlatinal hemolytic streptococci. The remaining case (10) was one of severe erysipelas. The precipitin reactions in this case, as in the four with findings indicating nonscarlatinal infection, were negative in both serums despite the fact that cultures revealed large numbers of hemolytic streptococci. No cases of scarlet fever developed in the school to which two of the patients (1 and 2) returned after the precipitin reactions with nasopharyngeal swabbings proved negative, and while they were still carriers of hemolytic streptococci.

In previous work [2b] I have shown that the scarlatinal antistreptococcus serum (H10) gave an almost negligible incidence of positive reactions with swabbings from persons ill with other diseases such as follicular tonsillitis, from those with diseases of the nervous system, and from normal controls. This was true likewise with this serum and the Dick concentrated antitoxin when swabbings were made in nineteen normal persons of similar age who were not exposed to scarlet fever during the course of the experiments recorded in the tabulation.

The fact that two contacts developed nonscarlatinal infections of the throat during the early part of the outbreak, and one during the latter, and that five of the patients developed scarlatinal infections, two with rash, during the middle period of the outbreak, suggests the possibility that the hemolytic streptococcus first acquired and then lost specificity and toxin producing power, during the course of this small epidemic.

SUMMARY

The close agreement between the specific precipitating power of the serum, prepared by the injection of suspensions and cultures of the hemolytic streptococcus from scarlet fever, and the antitoxic serum, prepared by the injection of filtrates or toxin of scarlatinal hemolytic streptococci, suggests that sufficient bacterial substance goes into solution in their cultures to make their antitoxic serum suitable for the diagnostic precipitin reaction.

GAS PRODUCTION BY BACTERIAL SYNERGISM

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When two bacteria living in association bring about changes which neither is able to produce alone, it is a little difficult to find a term to define this relationship. The subject of this paper is concerned with such phenomena in which gas production is the index of the changes and we have chosen the term "synergism" to express it. This term is widely used in pharmacology and indeed is to be found in theology where it indicates "the combined efforts of the human being and the divine grace in the salvation of the soul." It is of interest to note that this culmination cannot be reached by the single effort of either factor. Recently the term has been used in bacteriology by Kammerer [1] for an analogous process in the production of porphyrin in the intestinal tract and urobilin from bilirubin by bacterial cooperation between B. putrificus and certain undetermined aerobic bacteria. Symbiosis suggests mutual benefit which we are not convinced plays the dominant part in the phenomena we are considering. Metabiosis is too general in its scope to make clear what we wish to indicate. Synergism, as we use it, merely defines what occurs.

It must not be overlooked that under natural conditions this cooperative action must be extremely common as in the sulphur cycles, the breakdown of animal and plant tissues, the natural history of septic wounds (Goadby [2]), and probably in many other infections in man. Repression of biochemical characters has long been appreciated in the bacteriology of milk and the studies of the so-called sparing action of carbohydrates on protein. It is not difficult, therefore, to conceive that the reverse action also does occur and that one organism in a mixture may favor the manifestation of the activity of another. The gradual change in the reaction of a medium favors the growth in mixed cultures of certain forms and represses others. The growth of yeast appears late in a lactose medium when it is mixed with bacteria capable of fermenting the lactose to acid, and a delayed gas production occurs from the activity of the yeast. This has been used in our laboratory for isolating yeasts from the oral cavity. Conditions favoring growth also tend to favor the metabolic activity. Sometimes we depend for our

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isolations on encouraging the growth of certain bacteria; sometimes we recognize their presence in a mixture before isolation by a striking product of growth. This idea need not be further stressed since it is widely recognized in laboratory practice.

There is comparatively little to be found in the literature bearing directly on the subject of our report.

Marshall Ward[3] described a change produced by a yeast and a bacterium in a saccharine fluid containing ginger resulting in a preparation resembling ginger beer. It required the two organisms working together to obtain the result. Nencki[4] reported the formation from glucose of normal butyl alcohol by a mixture of B. paralactici and B. chauvoei, neither of these bacteria being able to produce it in pure culture. Burri and Stutzer[5] were able to demonstrate the development of free nitrogen from sodium nitrate by mixtures of B. coli and B. denitrificans which was not formed by either of these bacteria alone. The process, however, was easily followed in definite stages of reduction of the nitrate in this case. Marshall[6] has clearly demonstrated the stimulating influence of an alkali-producing organism (B) on the acid production of lactic acid bacteria (A) on the souring of milk. He further showed that other types of bacteria retard the souring process. Killed cultures of the bacterium (B) were as effective as the living, which would indicate phenomena quite different from those we are considering. We have no doubt there are many examples of such relationships which have not been correctly interpreted, both in laboratory cultures as well as in the process of disease production in the animal body.

Several years ago it occurred to one of us (W. L. H.) that the confusion of results in many fermentation tests could, not uncommonly, be explained by such mixed cultures. In the study of streptococci it was shown that mixed cultures are not always easy to detect and that the presence, in a culture of hemolytic streptococcus, of a streptococcus viridans may be readily overlooked.[7] Previous to this work on streptococci a preliminary study was carried out on the possibility of mixed cultures being the explanation of certain irregularities in fermentation tests with the colon-typhoid group of bacilli. It is obvious that the chances of picking cultures containing streptococci would be more frequent with these relatively large colonies. The results showed that if a lactose fermenting streptococcus were added to a culture of B. paratyphosus in a tube of lactose broth, gas was produced although no gas was ever formed in this medium by either microorganism in pure culture. The same result was obtained using a saccharose fermenting streptococcus and a B. coli communis (non-saccharose fermenter). Staphylococcus could be substituted for the streptococcus with similar results and a number of combinations were studied. In such mixed cultures the presence of the streptococcus is not always evident since the gram negative bacilli outgrow it in these serum free mediums.

Since about 1912 the students of bacteriology have each year had this source of error in the interpretation of the results of fermentation tests demonstrated to them. It was emphasized that: false readings could be made in the presumptive test for B. coli when dextrose gas producers apparently form gas in lactose in the presence of cocci capable of attacking lactose with acid production; colonies picked from Endo plates and apparently pure but actually containing lactose fermenting cocci may be discarded as gas producers in lactose when, in reality, B. paratyphosus may be present; in general studies, using such fermentation tests, a culture unknowingly mixed may give, on a retest some time later, results

entirely different if one of the bacteria has in the meantime died out. In 1916 J. E. Rush in our laboratory in Pittsburgh undertook a more detailed study of the conditions of the phenomenon but his results were never published and the work was not continued at the time. Our working hypothesis for these reactions was that the coccus or other non-gas producing microbe split the disaccharide, setting free monosaccharides which were then attacked by both bacteria in the mixture, gas being produced by the colon-paratyphoid group under certain conditions.

The report of Theobald Smith and D. E. Smith[8] on the inhibitory action of paratyphoid bacilli on the fermentation of lactose by B. coli has, we believe, a direct bearing on the problem. The phenomenon they described is the reverse of the one we are considering but nevertheless has to do with the intimate metabolism of bacteria acting in association. These authors noted a marked difference between the effect of the hog cholera strains and those of the paratyphoid bacilli. They found all grades of inhibition depending on the age of the cultures of the paratyphoid bacilli and that living, physiologically functioning, bacteria or those recently killed are necessary. The suggestion is offered that the inhibitory substance is some metabolic product of the paratyphoid bacilli, possibly an enzyme. Their experiments supported the theory that the acid-producing and gas-producing entities in cultures are distinct.

Sherman and Shaw[9] showed that the addition of Streptococcus lacticus or Lactobacillus casei to a culture of B. acidi-propionici in a lactose medium increased the amount of propionic acid six or seven-fold. Bacteria unable to ferment lactose gave similar results so that the theory of propionic acid being formed from the lactic acid produced by the added microbe was given up. This is a type of synergistic action although gas is not the criterion used.

A careful study on this subject was reported by Sears and Putnam.[10] They worked with a number of gas-forming pairs and used lactose, saccharose and mannit as the fermentable substances. The gas-producing bacteria in the mixtures were members of the paratyphoid group, the B. mucosus group, B. morgani and B. proteus vulgaris. A variety of non-gas but acid forming bacteria were used in combination with the above. The results were not altogether constant. It was unfortunate that these authors used B. proteus vulgaris in combination with a streptococcus for the more detailed part of their study because of the active proteolysis of this bacillus and the rather prompt and continuous alkaline changes which it produces. This complicated the picture, the hydrogen-ion concentration being decidedly lower for the combination growing together than for the streptococcus alone. They did not believe that the age of the cultures or the numbers of each bacterium added affected the results but an analysis of their tables shows a very decided effect for each of these factors. Both bacteria, they also demonstrated, must be growing together. Their explanation of the phenomenon was that the acid former degraded the sugar to a form utilizable by the gas former but that this degraded substance was not glucose.

A particularly interesting example of a bacterial association is found in the study of Speakman and Phillips[11] on the production of lactic acid. During the late war acetone and butyl alcohol were produced on a large scale by fermentation of cereals and other carbohydrates. Serious difficulties developed in the various plants due to the contamination of the cultures of B. granulobacter-pectinovorum with an aerobic bacillus named Bacterium volutans by Thaysen. The result of the mixed culture was a dropping off or complete absence of the production of acetone and a sharp increase in the lactic acid development. This increase was the result of an altered metabolism on the part of the acetone producer, resulting in a depression or loss of the acetone producing power and a stimulation of an

otherwise weak function of lactic acid production. The relative numbers of each bacterium added determined the results. The tentative hypothesis offered by these authors was that the inhibiting substance was a product of nitrogen metabolism of B. volutans in media with vegetable protein and only traces of carbohydrate material.

Castellani [12] overlooked the previous work on this subject and has recently reported a series of experiments using a variety of combinations similar to those already mentioned. He found that the gas producer must be capable of fermenting glucose with the formation of gas. However the reverse might not be true since not all glucose gas fermenters would give acid and gas in mannit in the presence of the acid producing B. typhosus although he cited no example of this phenomenon. Castellani considered the subject very obscure, which indeed it is, and apparently thought the observations were new.

The Problem.—Little need be added to what already has been said to emphasize the importance of further work on this phenomenon. It may be a source of error in identifying bacteria. It may explain certain supposed alterations or transmutations. It should add considerably to our knowledge of bacterial metabolism and especially of the stages in carbohydrate degradation. It may have a direct application to many problems in the study of infectious diseases and the processes normally occurring in the gastrointestinal tract.

BACTERIA WHICH GIVE RISE TO THE PHENOMENON

We first satisfied ourselves that gas can be produced by the following pairs in a carbohydrate medium in which neither of the bacteria can form gas when growing alone.

To these we may add the results of Sears and Putnam [10] and those of Castellani [12] which do not change the general scheme requiring that one of the pair must act on the substance of the test with acid production and the other must be able to ferment glucose.

There are so many possible combinations that it was difficult to decide which should be chosen. We finally decided on a Streptococcus fecalis recently isolated from an appendix removed at operation and a B. coli communis obtained from human feces. These bacteria were repeatedly plated and retested. They were culturally and biologically true to type. They both grew luxuriantly in the medium used which we considered important for the purpose of our studies and it was chiefly for this reason they were chosen. They formed gas when growing in association more constantly than many other pairs and the B. coli never attacked saccharose when growing alone. In all our experiments, control tubes using the same lot of medium were always separately seeded with each organism and kept under the same conditions.

BASIC MEDIUM

The basic medium employed was plain broth (0.3% Liebig's Meat Extract, 1.0% Bactopeptone and 0.5% sodium chloride) sterilized at 12 pounds pressure for twenty minutes and adjusted to give a final reading of P_H 7.4. Andrade's indicator was used in most of the tests in 1% amounts. Merck's saccharose in 10% solution in distilled water was sterilized separately and added to make a 1% solution to the plain broth. There are many difficulties in making these disaccharide mediums and great care must be exercised and frequent controls used to be sure that splitting of the sugar does not occur. We tried a number of other mediums but the results were unsatisfactory. "Hormone" broth would need to be fermented before it could be used in such a study since it

TABLE 1

BACTERIAL PAIRS WHICH IN ASSOCIATION PRODUCE GAS IN INDICATED CARBOHYDRATE

Pairs			Carbohydrate
Staphylococcus aureus	+	B. paratyphosus B	Lactose
Streptococcus fecalis	+	B. cholera suis	
Streptococcus fecalis	+	B. paratyphosus B	
Staphylococcus aureus	+	B. proteus vulgaris*	
Streptococcus fecalis	+	B. proteus vulgaris*	
Streptococcus fecalis	+	B. paratyphosus A	
Staphylococcus aureus	+	B. coli communis	Saccharose
Streptococcus fecalis	+	B. coli communis	
Streptococcus fecalis	+	B. acidi lactici	
Streptococcus ignavus	+	B. paratyphosus B	
Streptococcus ignavus	+	B. paratyphosus A	
Staphylococcus aureus	+	B. paratyphosus A	
Staphylococcus aureus	+	B. proteus vulgaris*	Mannit
Streptococcus fecalis	+	B. proteus vulgaris*	
Streptococcus pyogenes	+	B. proteus vulgaris*	
B. dysenteriae (Flexner)	+	B. proteus vulgaris*	
B. typhosus	+	B. proteus vulgaris*	

* Nonlactose fermenter and rapid liquefier of gelatine recently isolated from a case of chronic otitis media.

contains definite amounts of monosaccharides and we were particularly anxious to avoid the complications necessarily present if one uses B. coli or other bacteria for preliminary fermentations of the basic mediums. For this reason we did not use meat infusion mediums which Theobald Smith and D. E. Smith [8] used in their studies.

Tubes.—A modification of the Smith fermentation tube was generally used although Hall's modification of the Durham tube was found convenient in numerous preliminary tests. For quantitative readings of gas production, 5 cc. pipettes were bent into "U" tubes and the end sealed.

Optimum percentage of carbohydrate.—Theobald Smith and D. E. Smith [8] did not find the amount of lactose altered the quantity of gas

produced by B. coli provided it was not too little but, as our experiments are somewhat different, we tested out varying percentages of saccharose. The amounts ranged from 0.1 to 4% and the tubes were simultaneously seeded with B. coli and Streptococcus fecalis. Taking 10 as the maximum amount of gas produced, table 2 shows our results.

The comparative quantities of gas are only approximate and as there are so many factors involved in accurate measurements of gas production, we have not stressed small differences. Nevertheless, comparisons of the amounts of gas produced do give indications of the presence of favorable or unfavorable conditions. As a result of these tests we decided to use the saccharose in 1% amounts.

TABLE 2

Approximate Results of Varying Saccharose Content on the Gas Production by Streptococcus Fecalis and B. Coli, with 10 Taken as the Maximum

Percentage of saccharose....	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0	2.5	3.0	3.5	4.0
Gas production	1	2	3	4	5	6	7	8	9	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

THE TWO BACTERIA MUST BE LIVING IN CLOSE ASSOCIATION IN THE MEDIUM

Filtration of the cultures through Berkefeld filters.—Each bacterium as well as the two together were grown in the saccharose broth for 24 hours and then the cultures were filtered through Berkefeld filter candles. The reaction of the filtrates from the streptococcus cultures was readjusted. Each filtrate, after testing for sterility, was added to a fermentation tube and seeded with the other bacterium, the filtrate from the mixture being seeded with B. coli. There was no gas produced in any of the tubes. If an enzyme had been present it may have been adsorbed by the walls of the filter. The experiment would indicate that the hypothetical substance set free by the streptococcus from the saccharose is unstable or that for some reason it could not be used by the B. coli for gas production. It is possible that it also may have adhered to the filter candle.

Growing the bacteria in a "U" tube containing sand.—A long "U" tube was partly filled with thoroughly washed and ignited sea sand. It was then filled with plain broth to about one inch and a half above the level of the sand. The height of the sand was 24.5 cm. and the tube was about 1.5 cm. in diameter. It was plugged and sterilized. The streptococci and B. coli were added in equal amounts to the broth in one

arm of the tube. Transfers were made to plain broth from the fluid in the opposite arm after 40 hours' incubation at 1-hour intervals for 8 hours and then 24 hours later. Plain saccharose broth tubes were seeded from the broth, blood agar plates were streaked and smears were stained. The experiment showed that B. coli came through the sand in 40 hours. No change was noted in the saccharose until the streptococcus was also present in the subcultures. In one experiment this required 72 hours. Gas was then produced as usual.

A slightly different experiment was then tried. One arm of the "U" tube was plugged with a rubber stopper after filling the apparatus with the saccharose broth. Air was excluded from the stoppered arm and the tube somewhat resembled the other fermentation tubes except for the presence of the column of sand. The open arm was seeded as before with the two microorganisms. At the first signs of gas a subculture was made from the closed arm and streptococcus was found to be present. These experiments clearly indicate that the two microbes must be actually growing together and in intimate association. There is no evidence that the colon bacillus has been altered by the streptococcus and so takes on the function of fermenting the saccharose.

EFFECT OF DEAD B. COLI ON THE ACTIVITY OF STREPTOCOCCI

A heavy growth of B. coli on blood agar plates was killed by exposing it to formalin vapour. A suspension of these bacteria was added to the saccharose medium along with approximately the same amount of the living streptococcus. There was no inhibition of the growth of the streptococcus but at no time up to 96 hours was there any evidence of gas production.

EFFECT OF HYDROGEN-ION CONCENTRATION

Hydrogen-ion concentration plays a most important rôle in modifying bacterial growth and metabolism and therefore it must be of particular importance in the problems we are studying.

Three flasks of saccharose broth without indicator were seeded with three loops of an 18 hour culture of B. coli, Streptococcus fecalis and a mixture of the two respectively. These were incubated and tested at intervals by Brown's method [13] to determine the reaction. A typical average result is shown in table 3.

It will be seen that the B. coli culture shows a considerable degree of acidity after the first few hours and then a return to its original reaction

followed by a slightly alkaline change; Streptococcus fecalis remains at about the same hydrogen-ion concentration for 8 hours and then becomes frankly acid between the 8th and 13th hour; the mixture appears to be dominated by the B. coli at the beginning of the growth, but later develops acid due to the activity of the streptococcus finally reaching a reaction close to that of the streptococcus alone. The relation of growth curves to these changes is, as one would expect, very close.

STAGE OF GROWTH

The growth curves were established in the usual way by plating a series of dilutions of the various cultures after different periods of incubation. The period of lag is definitely different in the two microbes.

TABLE 3

Hydrogen Ion Concentration: Ph Values for B. Coli and Streptococcus Fecalis in Saccharose Broth

Time after Seeding in Hours	B. coli	Streptococcus fecalis	Pairs
1	7.4	7.3	7.4
2	7.3	7.2	7.3
3	7.1	7.5	7.2
4	6.7	7.3	6.8
5	6.7	7.4	6.7
6	6.7	7.4	6.7
7	6.7	7.4	6.7
8	6.7	7.3	6.7
13	7.2	5.8	6.6
26	7.7	5.7	5.9

The streptococcus takes approximately twice as long as B. coli to reach the peak of its growth curve. This is a very important factor in considering the combined metabolism of bacteria and has a direct application to our results as well as those of Sears and Putnam,[10] Theobald Smith and D. E. Smith [8] and Castellani.[12] The reports of Penfold,[14] Chesney,[15] Cullen and Chesney,[16] Anderson, Fred and Peterson,[17] Sherman and Albus,[18] Fred, Peterson and Anderson,[19] Bloomfield and Felty,[20] and many others have emphasized its significance in several fields. The investigations of Bordet,[21] da Costa Cruz [22] and others in the study of the abnormal metabolism of bacteria in the phenomenon of transmissible bacterial autolysis give further indications of its importance.

OPTIMUM AGES OF CULTURES

A striking difference having been noted in the lag periods of the growth curves of the streptococcus and the B. coli and realizing the practical impossibility of determining accurately the growth curves for

each bacterium when they are growing together, we decided to test the effect of mixing the cultures at different periods of their growth. Both bacteria were grown with daily transfers in saccharose broth for a week before the experiments. 1. The streptococcus culture of 24 hours' growth was kept constant in the first series of experiments and to it were added, every hour up to 14 hours, different amounts of the B. coli culture which varied in age from 4 to 17 hours. The results are shown in table 4.

It is seen that only when 1 cc. quantities of the B. coli culture were added to the 24 hour culture of Streptococcus fecalis did gas result. This amount of B. coli cultures 5 to 8 hours old gave constant positive results. These became irregular up to the 13-hour cultures and then

TABLE 4

GAS PRODUCTION (+) IN 24 HOURS WITH A STREPTOCOCCUS OF CONSTANT AGE AND B. COLI OF VARYING AGE

Time in Hours that B. coli Was Growing Before Inoculation	Amount of B. coli Culture Added to Tube of Streptococcus Culture		
	2 Loops to 1 Drop	1 Cc.	Control
4	0	0	0
5	0	+	0
6	0	+	0
7	0	+	0
8	0	+	0
9	0	0	0
10	0	+	0
11	0	0	0
12	0	0	0
13	0	+	0
14 to 17	0	0	0

remained negative till the end of the experiment. It was at first thought that with the inoculations of B. coli under 1 cc., death of B. coli had occurred due to the acidity produced by the streptococcus. This did not prove to be correct. On subculturing to blood agar plates, both bacteria were found to be viable and roughly in proportion to the amounts which had been added. When the negative results, with 1 cc. quantities of B. coli, were repeated, gas was produced. We were unable to determine the reasons for these variable results. It is clear that the quantities added play an important rôle and also the age of the culture has some influence. In connection with this series another experiment was tried. Each time 1 cc. of the B. coli culture of a given age was added to the 24 hour culture of the streptococcus, there was seeded into fresh saccharose broth 1 cc. of each culture. The result was that gas was produced in all the tubes but the amount was constantly less than

when B. coli was added to the full tube (15 cc.) of a 24 hour streptococcus culture.

The general trend of these results indicates that there is a changed metabolism in the relative activity of the two bacteria in attacking the products resulting from the preliminary breakdown of the sugar molecule. When relatively few streptococci are present this preliminary breakdown does not result in a sufficient amount of the hypothetical intermediate substance to satisfy the combined gas-producing action of B. coli and the acid production by the streptococcus, and the result is a lessened gas production. If, on the other hand, the streptococci are relatively vastly more numerous, this substance is formed in excess and more gas is produced. Further, if the B. coli are too few in number, as in 2 loopfuls or 1 drop in table 4, the gas-producing activity of the B. coli never comes into play. The B. coli also produces acid in the first stage of its fermentation of this intermediate substance as it does

TABLE 5

RESULT OF SEEDING UNEQUAL QUANTITIES OF STREPTOCOCCUS AND OF B. COLI TO SACCHAROSE BROTH

Streptococcus, 24 Hour Culture		B. coli, 14 Hour Culture		Result
15 cc.	+	1 cc.	=	Gas +++
1 cc.	+	15 cc.	=	Gas +

in its attack on glucose and evidently this stage precedes in time the gas-producing stage. Such an hypothesis more readily explains our results. In the growth curve there are stages where the bacteria are rapidly multiplying and physiologically most active. Their absorptive quality is high at this time. The stages of decreasing activity follow until growth ceases and the bacteria are latent. The balance between these stages in mixed cultures is extremely difficult to follow and many factors may interfere.

Having determined approximately the most favorable age of the B. coli culture, we carried out another series of tests using a B. coli culture from 10 to 18 hours old and adding the streptococcus cultures after different periods of growth, beginning with a 12 hour growth and then each hour up to the 24 hour culture. The results were most irregular, many tubes showing no gas and others varying amounts. There appeared a slight tendency, however, for the younger streptococci to be more active in helping the B. coli in its gas production. The test was repeated and every tube showed approximately the same amount of gas. Such

results show the inherent difficulties in controlling the innumerable factors which must be involved in an investigation of this nature.

ADDITIONS TO CULTURES AFTER GAS PRODUCTION HAS STOPPED

We have observed in numerous tests that gas production, under the conditions of our experiments, reaches its maximum after about 24 hours and thereafter remains practically stationary. Mixtures of the two organisms were made in a series of 52 saccharose broth tubes and after 48 hours, when the gas production was stabilized and approximately equal in all the tubes, a number of additions were made. Ten of these

TABLE 6

RESULTS OF ADDITIONS TO TUBES IN WHICH THE GAS PRODUCTION FROM THE MIXTURE OF STREPTOCOCCUS FECALIS AND B. COLI HAD STOPPED

Solution	Amount	Time. Hours	Tubes 1	2	3	4	5	6	Remarks
10% dextrose	1 cc.	24	0	0	0	0	0	0	
		48	d	d	0	d	0	d	
10% saccharose	1 cc.	24	0	0	0	0	0	0	
		48	d	d	d	d	d	d	
48 hour B. coli culture	1 cc.	24	0	0	0	0	0	0	The 6 tubes, duplicates
		48	d	d	d	d	d	d	
Physiological salt sol.	1 cc.	24	+	+	+	0	+	+	
		48	+	++	0	0	+	+	
	5 cc.	24	0	+	+	0	+	+	Compression of gas by weight of fluid must be considered
		48	0	+	+	0	+	+	
Sodium Hydroxide	N/20 in amounts to give N/1 in cc.		(0.005)	(0.01)	(0.015)	(0.02)	(0.025)	(0.03)	
		24	0	0	0	0	+	+	
		48	0	0	0	0	+	+	
	N/1 in cc.		(0.05)	(0.1)	(0.15)	(0.2)	(0.27)	(0.3)	
		24	0	+	+	+	+	+	
		48	d	d	+	+	++	d	
Controls (no additions)		24	0	0	0	0	0	0	(10 tubes) alike
		48	d	d	d	d	d	d	

+, 0, and d indicate respectively, increase, no change, and decrease of gas.

tubes were used as controls and the remaining 42 were divided into seven lots of six each. The amount of gas was noted at the time of the additions and records were kept of any increase or decrease from this amount. The results are shown in table 6.

There are many difficulties in interpreting such results but it must be remembered that we are dealing with biological phenomena in which strict regularity is hard to obtain. The reactions, whatever they are, have apparently come to a standstill since the addition of a new supply of dextrose or saccharose did not give any different result from that of the controls nor did a further addition of B. coli have any effect.

The addition of 1 cc. of physiological salt solution gave an increase in gas in five out of the six tubes in 24 hours and a further increase in one tube after 48 hours. That the dilution is not the only factor in this result would be suggested by the failure to get the same response by the addition of equal quantities of fluids containing carbohydrates and B. coli. The effect on the hydrogen-ion concentration must be negligible on account of the buffer action of the medium although the buffer content was purposely kept low. Holm and Sherman,[23] and Sherman and Holm [24] have reported that salts which accelerate growth of B. coli seem to widen the zone for hydrogen-ion tolerance. They found 0.20 M sodium chloride increased the rate of growth over that obtained in 1% peptone at all P_H values but most markedly at the acid range of P_H 5.2 and Winslow and Falk [25] found that sodium chloride at a strength of 0.0145 M had a distinctly favorable influence on the viability of B. coli in water at a P_H of 6.0 which is roughly in the vicinity of the reaction we are considering (table 3). It is probable that the sodium chloride concentration becomes less in these cultures and the added saline supplies the loss. The addition of greater amounts of the same saline did not have as constant a result. The additional weight of fluid in the open arm may have favored some gas absorption and masked any additional gas which may have been formed.

Alkali addition is seen to have a very definite stimulating effect according to the amount added. With the lesser amounts of alkali the only change is the absence of decrease in the 48 hour period suggesting the activity of the streptococci in preventing the normal alkali production by B. coli which would lead, if it occurred, to the absorption of gas as in the controls. There is some irregularity in the range from 0.025 cc. to 0.1 cc. This may be due to the slower diffusion in the tubes receiving the undiluted normal solution. The addition of between 0.27 cc. and 0.15 cc. seems to be most effective. In the tube with 0.3 cc. the increase is followed by a decrease. It must be considered that in all these tubes the addition of alkali will favor the absorption of the carbon dioxide from the gas already formed so that the increase may be greater than indicated.

In this connection another experiment was devised by substituting dextrose for the saccharose in the fermention tubes. Three tubes of dextrose broth were prepared. Two of these were seeded with Streptococcus fecalis and one with B. coli and incubated for 24 hours. The B. coli tube showed copious gas formation; the two streptococcus tubes were frankly acid. Both of these latter were seeded with B. coli and at the

end of 72 hours when no gas was evident, a little saline was added to one and normal sodium hydroxide to the other. At the end of 24 hours gas was present in both tubes.

THE EFFECT OF ALKALI-PRODUCING BACTERIA

The idea suggested itself that if the acidity plays such an important rôle in the phenomenon, the addition of an alkali-producing bacillus might favor the gas production. However, after repeated trials of various combinations using B. alkaligenes as the alkali producer, it was found that no beneficial results were obtained. It is quite probable that the normal metabolism of B. alkaligenes is interfered with by the acid reaction produced by the streptococcus. The use of B. proteus as shown by Sears and Putnam [10] gives excellent results and the factor of alkali production by this bacterium is undoubtedly of importance. It is further significant that Theobald Smith and D. E. Smith [8] worked with cultures giving a marked final alkalinity and it would be interesting to know what the reaction curves in point of time were in their two groups. They suggest that the alkali maximum is in the vicinity of 18 days for the hog cholera group and probably at about 4 days for the paratyphosus group. It is quite possible that as acid interferes with gas production, so also may alkali, but this alkali must be actually being formed in close association with the second bacillus as will be discussed later. The cultures of B. paratyphosus becoming older, the inhibitory action disappeared probably due to loss of metabolic activity.

ORGANIC ACIDS IN DOUBLE CULTURES

Koser [26] has shown that certain organic acids can be utilized by members of the colon-aerogenes group which attack them in the form of the sodium or other salts and produce an alkaline reaction. That some such process may be responsible for the results obtained in acid mediums with B. coli and that this function is dominant under certain conditions, and the gas-producing function is dominant under other conditions, may very well explain the findings. If a colon bacillus finds its carbon more available in the form of the organic acid salts (which undergo electrolytic dissociation to a much greater extent than the acids), it may very well spare the carbohydrate. It was shown by Grey [27] that formic acid is an intermediate product in the decomposition of glucose by B. coli and that the formic acid itself is attacked in a secondary fermentation. The fermentation of a carbohydrate is therefore more complicated and probably takes longer, although Ayers and Rupp [28] believed the two

actions to be simultaneous. The evidence of many investigators supports the view that acid production and gas production are separate and probably due to different enzymes. Formic and other organic acids are certainly formed by the Streptococcus fecalis. Langwill [29] has shown that non-hemolytic streptococci produce formic and acetic acid, hemolytic streptococci and Streptococcus viridans, acetic and propionic but no formic acid. Foster [30] had previously reported a hemolytic streptococcus which produced chiefly lactic acid in glucose broth, less acetic and a trace of formic. Many years before Sieber-Shoumoff [31] reported that certain streptococci form the optically active while others form the inactive lactic acid. Little is known about the most favorable hydrogen-

TABLE 7

EFFECT OF AMMONIUM LACTATE AND DEXTROSE ON THE H-ION CONCENTRATION AND GAS PRODUCTION OF THE BACTERIA

Medium; Plain Broth Containing 1% of Each	Culture	Time of Reading: 20 Hours		41 Hours		67 Hours		113 Hours	
		P_H	Gas in Mm.	P_H	Gas in Mm.	P_H	Gas in Mm.	P_H	Gas in Mm.
Ammonium lactate.....	B. coli	6.8	0	7.1	0	7.3	0	7.3	0
Ammonium lactate.....	B. coli	6.8	0 Streptococcus fecalis added	7.1	0	7.5	0	7.5	0
Dextrose.....	B. coli	5.0	30	4.8	24	5.1	24*	4.9	24*
Dextrose.....	B. coli	5.0	22 Streptococcus fecalis added	4.9	20	4.8	20*	4.8	20
Dextrose + ammonium lactate	B. coli	5.7	13	5.3	12	5.5	12*	5.3	12*
Dextrose + ammonium lactate	B. coli	5.7	3 Streptococcus fecalis added	5.5	7	5.1	7*	4.9	7*
Ammonium lactate.....	0	6.9	Streptococcus fecalis added	6.9	0	6.9	0	6.9	..
Ammonium lactate.....	0	6.9	.....	...	..	...	..	6.9	..

* Slight absorption of gas.

ion concentration for the reactions with the salts of organic acids. Koser [26] used in his experiments a P_H of about 6.7.

With these ideas in view, we undertook to test in a general way the possible effect of the salts of the organic acids on the metabolism of these double cultures. Two tubes containing 1% ammonium lactate, two containing 1% dextrose, two containing 1% dextrose plus 1% ammonium lactate were seeded with a B. coli culture about 8 hours old. P_H of all tubes was 6.9. At the end of 20 hours' incubation, there was no gas and a P_H of 6.8 in the ammonium lactate tubes, marked gas in the dextrose tube at a P_H of 5.0, and much less gas with P_H of 5.7 in the tubes containing both dextrose and ammonium lactate. A 20 hour culture of Streptococcus fecalis was then seeded in the second tube of each

pair and the results were read 21 hours later. The results are seen in table 7.

No gas is formed directly by either or both organisms from ammonium lactate but there is an increase in the alkalinity from P_H 6.8 to P_H 7.3 with B. coli and to P_H 7.5 with the mixture. Streptococcus fecalis alone did not change the reaction. There is a lessened gas and acid production by B. coli when ammonium lactate is added to dextrose in the medium. The addition of streptococcus to the B. coli culture in dextrose tends to somewhat increase the acidity.

It would appear that the action of B. coli on dextrose and ammonium lactate may be simultaneous as Ayers and Rupp [28] have shown and that the presence of the ammonium lactate interferes with gas production from the dextrose. The addition of streptococci to this lagging gas production appeared to increase the gas in one instance and showed a definite tendency to increase the acidity. Ayers and Rupp had the same general results using dextrose with acetate or formate, that is, the reversion to alkaline reaction was more rapid in the presence of the formate and they considered this due to the oxidation of the salts to carbonates. These authors, it should be recalled, use a medium in which the only source of nitrogen for the bacteria was sodium ammonium phosphate and that in our experiments we used ordinary broth. In our results the deaminization with ammonia production must be also considered in the neutralization of the acids.

We propose to repeat this experiment with different time periods for adding the two bacteria and using other salts of the organic acids such as the formates and acetates. It was tried as a preliminary test in the search for a possible explanation of the phenomena and will be considered in the discussion.

NATURE OF THE GAS PRODUCED

If the process is similar to that which occurs in the fermentation of dextrose by the B. coli, the ratio between the hydrogen and carbon dioxide produced should be very similar. Theobald Smith [32] determined the gas ratio for 15 strains of B. coli as approximately $\frac{H}{CO_2} = \frac{2}{1}$, using dextrose and lactose. For saccharose it was about $\frac{H}{CO_2} = \frac{3}{2}$.

To determine the correctness of this view, we seeded saccharose broth with the streptococcus and the B. coli and when gas production was at its maximum, we analyzed the gas by the alkali absorption method. The gas showed approximately 85 to 80% hydrogen and 15 to 20% carbon

dioxide. The B. coli alone in a dextrose broth gave a ratio of about 60% hydrogen and 40% carbon dioxide. The synergistic gas ratio was $\frac{H}{CO_2} = \frac{4}{1}$, the B. coli ratio $\frac{H}{CO_2} = \frac{1.5}{1}$, the Theobald Smith ratio being $\frac{H}{CO_2} = \frac{2}{1}$. This indicates that the process is different in the two cases and would suggest some loss of the carbon dioxide, probably in the form of carbonates as Ayers and Rupp [28] have shown. This could be definitely determined by estimating the total amount of CO_2 in the medium as compared with controls but was not done by us. It is not clear whether it is on dextrose as an intermediate substance that the alteration takes place or at a lower step as in the formic acid stage of the fermentation, but it is probable that the whole metabolic balance is changed.

IS DEXTROSE PRESENT?

Use of polarimeter and saccharimeter.—We endeavoured to throw some light on this point by the use of polarimeter and saccharimeter measurements but the instruments available were not delicate enough to record any appreciable change, the loss of saccharose under these conditions being comparatively slight.

Examination for reducing substances.—Repeated attempts to demonstrate the presence of a reducing substance at varying periods of the combined or individual growths have all resulted in failure. Benedict's method was employed. The glucose may very well be split off from the saccharose molecule but it is probably immediately utilized by either or both bacteria in the production of various organic acids. We had no difficulty in demonstrating that saccharose is still present in large amounts after the Streptococcus fecalis has been growing in the medium for up to a week. No attempt was made to determine the amount of saccharose quantitatively.

Formation of osazones.—The facts that saccharose is not reduced by Fehling's solution and does not form either hydrozone or osazone crystals when treated with phenyl hydrazine lead us to attempt to discover if osazone could be demonstrated from the action of Streptococcus fecalis in the saccharose medium. A suspension was made of living streptococci in physiological salt solution and divided in two test tubes. One tube was boiled to kill the microbes; the other was untreated. Each tube was then added to plain saccharose broth, and chloroform in excess amounts was immediately poured in and the mixture thoroughly shaken. At the end of 24 and 48 hours samples were removed, the phenyl hydra-

zine reagent (phenyl hydrazine hydrochloride and sodium acetate) added and the tubes boiled. It was found that the culture of living streptococci yielded much more profuse osazone crystals than could be found in the control with the killed bacteria. This would suggest the presence of a saccharose-splitting enzyme but the saccharose may have been partially split by the boiling in the presence of slight amounts of acids, possibly formed in the medium by the streptococci before the chloroform had become effective. However, the sodium acetate used should effectively buffer such an acid if formed. The technique will need to be greatly improved, more delicate tests for glucose should be employed and we plan undertaking a more careful study of this phase of the problem.

DISCUSSION

The word "synergism" seems an appropriate term to define all this class of phenomena in which the biochemical reactions brought about by two or more microorganisms in association are different from that produced by any of them growing in pure culture. The phenomena we are considering which concern the fermentation of carbon containing compounds such as the carbohydrates and their corresponding alcohols, should all be grouped under one heading. There is a slight suggestion of mutual benefit in some of the experiments but it appears to take a very minor place in the final results. The inhibition of gas production in the examples given by Theobald Smith and D. E. Smith,[8] and Speakman and Phillips [11] are due fundamentally to changes in the metabolism of the bacteria analogous to that which occurs in the increased gas production of our studies.

The problem is far broader in its scope than can be fully discussed in the limits of this paper since it includes, as cited in the work of Kammerer [1] and his associates, phenomena of combined proteolytic activities, the finer chemistry of the carbohydrates and a far fuller knowledge than is possessed today of the more intimate metabolism of microbes. The difficulties of analyzing the processes where two microorganisms are growing in association are easily appreciated if one considers how little information is available on the more simple reactions with single forms.

The confusion which mixed cultures bring to the study of bacteria is generally recognized by all, but it still needs continual reemphasis and the examples discussed in this paper should further stress the importance of working with really pure cultures. Fermentation tests are practically essential for the isolation and identification of many groups of bacteria

and the possible errors in their use should be well known. In water analyses, in plating from stools, in the identification of the supposedly isolated cultures, in the possibility of later modifications or transmutations, the phenomena we are considering play a most important part.

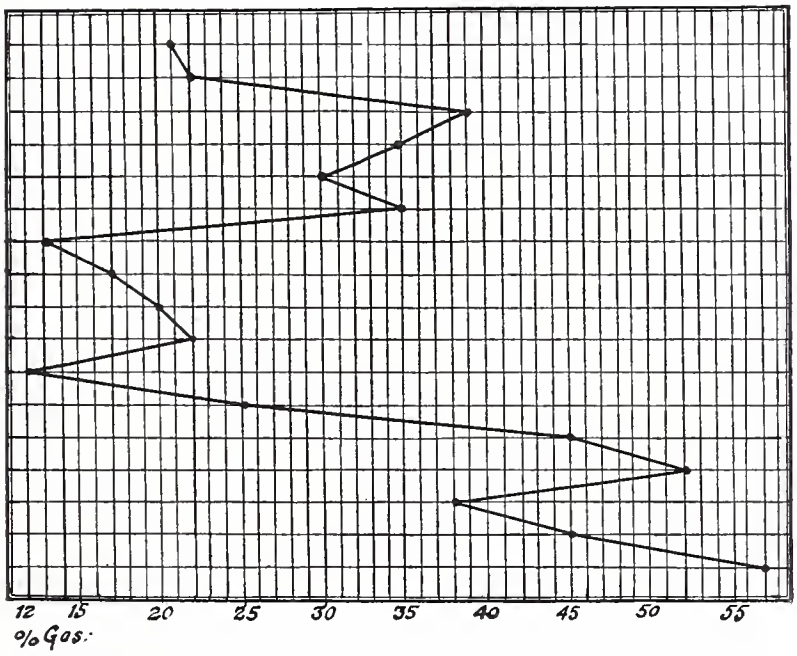
Many combinations give the confusing results of synergistic activities. These may be morphologically similar as where B. typhosus and B. proteus are concerned or entirely different when a coccus is the second microbe. The first mixture will lead to greater confusion than the second but due to the more active growth of the colon-typhoid bacilli in the media used, the presence of cocci may also be readily overlooked.

The two bacteria may be present in the mixture and yet not give rise to the phenomena. If for any reason the medium favors the multiplication of the acid-producing member of the pair up to a certain stage, the gas-producing bacillus will fail to produce gas. This is shown in our experiments with a streptococcus and B. coli in which the streptococcus inhibited the gas production by B. coli when it was cultured for a short time in dextrose and saccharose broth before the B. coli was added. The results of Theobald Smith and D. E. Smith,[8] moreover, demonstrated that if B. paratyphosus were grown for some days in lactose broth, the B. coli added later did not produce gas in this sugar so characteristically attacked by it. It is readily appreciated that these conditions can obtain if, for example, the gas-producer is in relatively small numbers or is temporarily checked in its early growth. The manifestation of the mixture also may be greatly delayed as we have indicated in our finding that yeasts in mixed cultures in lactose broth only produce gas after many days' incubation.

Various grades of the phenomena can be obtained. It is often difficult to duplicate these conditions experimentally, using pure cultures accustomed to growing in the laboratory mediums, but it is comparatively easy to show that various grades of the reaction do occur. In the Theobald Smith and D. E. Smith [8] studies it was shown that the length of time the inhibiting paratyphoid bacillus was growing before the B. coli was added determined the amount of inhibition. The same is true in many of our experiments where cultures of different ages were added as shown in Table 5, and in the experiments to determine the optimum age for maximum gas production for the cultures used (table 4). Speakman and Phillips [11] also had similar results. It is to be noted that Sears and Putnam [10] showed in their tables the same kind

of gradation as will be seen from a study of chart 1, although they overlooked its significance.

When 100 streptococci were added to 300,000,000 B. proteus, the maximum amount of gas was produced. As the ratio between the organisms approached unity the amount of gas dropped, being at the minimum with 100,000,000 streptococci and 300,000,000 bacilli. With the increase in the numbers of streptococci to 10,000,000,000, little change was at first shown but as the numbers of the bacilli diminished to



Number of Streptococcus fecalis	Number of B. proteus vulgaris
10,000,000,000	3
10,000,000,000	30
10,000,000,000	300
10,000,000,000	3,000
10,000,000,000	30,000
10,000,000,000	300,000
10,000,000,000	3,000,000
10,000,000,000	30,000,000
10,000,000,000	300,000,000
1,000,000,000	300,000,000
100,000,000	300,000,000
10,000,000	300,000,000
1,000,000	300,000,000
100,000	300,000,000
10,000	300,000,000
1,000	300,000,000
100	300,000,000

Fig. 1.—Showing the effect on the gas production of altering the relative numbers of each bacterium.

(Prepared from Sears and Putnam's,[10] table 4)

300,000, there was a second rise which, however, never reached the height of the reverse ratio and as the numbers of the bacilli became minimal (30 and 3) another drop occurred. Since these readings were made after a ten-day period and the bacteria were presumably at the same stage of growth when added, it would appear that the streptococci in large numbers have a definite influence in changing the combined metabolism. We will shortly discuss some of the conditions for such results, such as the acid produced by the streptococci checking the growth

of the bacilli and the corresponding alkali production by the B. proteus tending to balance it. With this simple explanation the chart becomes tolerably clear. When streptococci are few they break down the mannit with a final acid-production but this acid is continually being neutralized by the products of the proteolytic metabolism, or by the alkali formed from the organic acids, both resulting from the activity of the large number of bacilli which are present. This favors the continued action of the cocci to attack the mannit and also the ability of B. proteus to further degrade to gas the intermediate bodies thus formed from mannit. As the number of cocci increases, there is an overproduction of acid and the cocci, being in greater numbers, attack the intermediate bodies to the relative exclusion of the fewer bacilli. This process continues until the bacilli, being reduced in actual numbers, grow rapidly in the less crowded medium, soon reach large numbers, and being more active in this stage of rapid growth, the neutralization and their increased metabolism tends to somewhat increase gas production but the more favorable conditions present when the slow-growing streptococci are few does not obtain. The bacilli added being still further reduced to a minimum (30 and 3) their multiplication fails to bring about a sufficient increase to prevent the retarding acid production by the predominating streptococci.

The two bacteria must be living in close association. This has been shown by Theobald Smith and D. E. Smith [8] and Speakman and Phillips [11] to be true for the inhibition phenomenon and Sears and Putnam [10] and we ourselves have found it to be true for the synergistic gas production. We demonstrated this fact by means of filtration, the "U" tube sand experiments, the modified fermentation tube with sand and the use of bacteria killed by formalin vapour. Unfortunately we did not test the effect of heating as Theobald Smith and D. E. Smith [8] did. These authors found that when the bacteria were killed at the minimum temperature, the inhibition was not entirely destroyed but it gradually disappeared if these same recently killed bacteria were incubated for a day or two and it also disappeared as the temperature used for killing was raised to 100 C. and over. These findings suggest that the bacteria must be physiologically active and that even after death of the organisms, some absorbing function may still be possible. This is based on the hypothesis that in our examples the two bacteria absorb, in proportion to their activity, the intermediate substance and, acting on it, produce acid or acid and gas, but if their environment is unfavorable the gas-producing function may be in abeyance. In the Theobald Smith

and D. E. Smith [8] experiments, the interference in gas production may have depended on the absorbing activity of the paratyphoid bacilli for the intermediate product set free from the lactose by B. coli and the metabolism of the same by the paratyphoid bacilli. If this metabolism is sufficient, the hypothetical substance is used up and none is available for gas production by the B. coli which apparently uses this same substance for its own acid production. Further, the paratyphoid bacilli may, probably largely by its alkaline products, make the environment such as: to interfere with the gas-producing power of the B. coli; to reduce the gas production; and to make the medium more soluble for the gas produced since alkaline medium holds in solution more gas than acid medium. It would seem that the bacteria recently killed by heat are still sufficiently absorbent to take up enough of this intermediate substance to lessen the inhibition and that this absorbing power is lost as the cell wall is more and more altered by heat and age. This is a very difficult matter to prove or disprove experimentally but numerous investigators have found the same principle of physiological activity to be important in other fields of bacteriological study such as those of bacteriophage. Here also dead bacteria may lessen the activity of the lytic principle presumably by absorbing it and, being unable to reproduce it, the amount is lessened.

The hydrogen-ion concentration plays an important part. The effect of hydrogen-ion concentration in these phenomena is evidently very great. In our experiments it is seen that gas production by B. coli is checked by the increased acidity from the streptococci and that the two bacteria reach their maximum acidity at different periods in their growth curves. We have shown that alkali will restart the process when this has stopped and, further, that it will allow gas production when this is held in check by the acid present. The addition of sodium chloride also gives an increase in gas production which may be due to the beneficial effects on the metabolism of B. coli as shown by Holm and Sherman,[23] and Winslow and Falk.[25] It may further lower the solubility of the medium so that more gas collects in the side arm. It is significant that in the experiments by Theobald Smith and D. E. Smith [8] the production of alkali was different in point of time in the two related microbes, the inhibiting paratyphoid bacilli being more rapid in producing alkali than the noninhibiting hog cholera bacilli. This is not strictly correct if we recognize that the group of B. paratyphosus (A) are also slow alkali producers and the B. dysenteriae (Flexner) group rapidly produce alkali and yet the B. paratyphosus (A) inhibited, and B. dysenteriae

did not. This phase of the subject will require careful study to determine if the inhibition is not a graded one. The acid production which always precedes the gas production changes the hydrogen-ion concentration and it may well be that the gas-forming enzyme requires for its activity this increase in acidity.

The reversion to an alkaline reaction and attack on organic acids: It is known that the reversion to an alkaline reaction exhibited by some cultures is due largely to the oxidation of the salts of the organic acids to carbonates and bicarbonates. It is also established that the organic acid salts are readily attacked by B. coli, the paratyphoid group and other bacteria with alkali production, but under favorable conditions gas may be formed, as Karczag and Schiff [33] have shown. These authors found that B. coli, B. paratyphosus B, B. enteritidis (Gaertner), B. typhi murium and B. pneumoniae ferment pyroracemic acid to gas. B. coli gave a gas which was 90% hydrogen and 10% carbon dioxide. These same organisms gave a hydrogen fermentation from formic and glycollic acids which indicated that these simpler acids are stages in the fermentation of the pyroracemic acid. Glycollic acid in broth gave no fermentation with B. coli but if a trace of pyroracemic acid were added, an induced reaction was started and prompt gas (not absorbed by alkali) was produced. A similar test with acetic acid and a trace of pyroracemic gave no gas. They gave the following scheme for the process:

CH_3CO		=	CH_3COOH		:	CH_3	=	CH_2OH
COOH			+			+		COOH
Pyroracemic acid			HCOOH			COOH		Glycollic acid
			Acetic acid Formic acid			Acetic acid		
CH_2OH			CH_2OH			CH_2OH		HCOH
COOH	+ H_2	=	O	+ H_2O	:	O	=	HCOH
			C			C		
			H			H		
Glycollic acid			Glycollaldehyde			Glycollaldehyde		Formaldehyde
HCOH			HCOOH			HCOOH		
HCOH	$+ 2H_2O$	=	HCOOH		:	HCOOH	$+ 2H_2 = 2CO_2$	$+ 2\ H_2$
Formaldehyde			Formic acid			Formic acid	Carbon dioxide	Hydrogen

This type of induced reaction is very important in considering the combined metabolism of two organisms. One bacterium may produce a substance in relatively small amounts, the presence of which changes the ability of the other to attack a given material which it would otherwise not do. It makes at least more logical the interpretation of many of the reported results.

Grey [27] believed that calcium formate is split by B. coli into calcium carbonate, carbon dioxide and hydrogen. Ayers and Rupp [28] have shown

that the rate of fermentation of dextrose and citrate varies with different cultures. The acids formed from the dextrose are turned into salts by the alkali in the media and the carbonates which are formed from these organic acids. The hypothetical substances from the carbohydrates which we are considering may therefore be degraded to organic acids; salts are formed which may in turn be changed to carbonates, and these may neutralize the acid. The evidence to be gained by the P_H determination may thus give a wrong impression of the actual processes. For better analyses of these phenomena the ability to attack the salts of the organic acids should be determined for the individual bacteria.

We purposely chose a strain of B. coli for our second culture which did not show a very marked secondary or eventual alkalinity in the cultures. The alkaline change in carbohydrate free mediums is chiefly due to ammonia. Sears and Putnam [10] used B. proteus and the alkalinity produced by the proteolytic activities of this organism may help to continue the process of gas production; otherwise we see no reason why they used 10 days as the time for reading their results. Our attempt to use B. alkaligenes with B. coli to test the effect of the alkaline production gave no better results than without it. Sears and Putnam [10] obtained more gas with those bacteria producing alkali as part of their known metabolism in the order given below. The substances tested and the percentages of gas are in brackets. L = lactose. S = saccharose. M = mannit. Streptococcus fecalis was used as the acid producer except where noted. B. cholera suis (L.5), B. coli communis (S.12), B. acidi lactici (S.15), B. proteus vulgaris (M. 16), B. pneumoniae (L.24), B. proteus vulgaris with streptococcus hemolyticus (M.26), B. paratyphosus B (L.30), B. proteus vulgaris with Streptococcus viridans (M.50).

We believe that the fact that six times as much gas was produced with B. paratyphosus B than with B. cholera suis has a direct bearing on the results obtained by Theobald Smith and D. E. Smith.[8] Our own results (table 7) demonstrated that when ammonium lactate was added to the dextrose broth there was less acid and less gas formed due to the carbonates which resulted from the attack on the organic acid salt by the B. coli. It has been shown especially by Ayers and Rupp [28] that the two phenomena, alkali production by attack on the organic acids and the splitting of the sugars with acid production, are simultaneous. The important differences are due to relative rates of these different actions. The B. aerogenes group attacks the organic acids much more readily than the B. coli group as Koser [26] and others have demonstrated.

That more gas is produced by B. aerogenes than by B. coli illustrates in single culture the same process as we believe is going on in our double cultures. It would appear that the carbonates are used up more readily in neutralizing the acids formed by the streptococci in the combined cultures and less is given off as gas. Thus, we found that the CO_2 production is markedly less in the synergistic cultures than in those of B. coli alone. In the former the ratio was $\frac{H}{CO_2} = \frac{4}{1}$; in the latter it was $\frac{H}{CO_2} = \frac{1.5}{1}$. A similar but more extreme ratio between carbon dioxide and hydrogen $\frac{H}{CO_2} = \frac{9}{1}$ given by Karczag and Schiff [33] using organic acids as the fermentable substance, is of interest here.

It would appear that in the results of Theobald Smith and D. E. Smith [8] the paratyphoid bacilli were more active in producing carbonates than the hog cholera bacilli and they may in their metabolically active stage, use up the available organic acids set free by the B. coli at a faster rate than the B. coli can use them to produce gas. Thus they inhibit gas production. There are probably also differences in the nitrogenous metabolism in the members of these two divisions of the paratyphoid bacilli.

In our own experiments we found that no gas was formed by the B. coli in the ammonium lactate broth and, further, that this organic salt added to a dextrose broth lessened the amount of gas and acid production. It is probable that the right proportion of available organic salts might also not only decrease but completely suppress gas formation. However, it is clear that the organic salts are not readily available for gas production after they are more or less stabilized and if the fermentable substance is in too great an amount, an induced reaction apparently does not result. Many things indicate that for gas production to occur, the products from the degradation of the carbohydrates must be well ionized or in a definite molecular form. This is shown by our filtration experiments where, although the organic acids are present from the streptococcus fermentation, no gas is formed from these by the B. coli. When the two bacteria are growing in the same culture the various intermediate products are actually being formed and are apparently in a condition to be readily available to the bacteria concerned. The association of lactic bacteria and oidium lactis in milk is somewhat similar. In this case the bacterium ceases to grow after a certain amount of acid is produced but the oidium destroys the acid and growth continues. Marshall [6] found an alkali producing bacterium assisted the lactic acid bacilli in producing acid.

In looking for a possible explanation of the steps in the processes, we are confronted with many unknown phenomena in the mechanism of carbohydrate fermentations. Whatever compound we are considering, the complications involved are equally great. Saccharose is believed to split into glucose and fructose; lactose into glucose and galactose; mannit, the alcohol corresponding to d. mannose, into mannose. These products if they exist as such in the early stage of bacterial fermentation are all readily attacked by the gas-producing bacteria we are considering. Glucose gradually partly changes in alkaline solution to d. fructose and d. mannose. "Hence a fermentation test obtained with glucose cannot be ascribed to that substance alone, but may have arisen from one or both of the other sugars." [34] We are therefore unable to follow why Sears and Putnam ruled out the simpler explanation of glucose being the mother substance of the gas because mannit gave abundance of gas. It may not be glucose but since all these monosaccharides, glucose, fructose and mannose are readily fermented to gas by the bacteria studied, it would be difficult to distinguish between them.

Fischer,[35] studied the acid curve of mixtures of B. coli and B. paratyphosus in lactose medium and found the curve steeper but not quite so high for the mixture than for B. coli alone. He believed it was due to a preliminary hydrolysis of the lactose, the product of which (monosaccharides) both bacteria used. It is true that the presence of these monosaccharides in the test tube cannot be proved but since they must be immediately attacked by both types of bacteria this is not surprising.

In a recent report Fernbach [36] and his associates have brought evidence to support the view that the molecular structure of the products from the hydrolysis of saccharose at the time the hydrolysis has taken place, is different from that which it assumes later.

Marshall's findings [6] would suggest that his bacterium B split the lactose in the milk and hastened the acid production and due to its alkali production steadied the process and allowed a greater amount of acid to be produced.

Some of the factors relating to enzyme activity have been discussed in considering the effects of the hydrogen-ion concentration. The hydrions activate certain enzymes and hydroxidions others. The presence of electrolytes on enzyme action is also important and this may explain the beneficial effect of sodium chloride when it is added to a culture in which gas production has ceased. Ptyalin loses its activity in the absence of sodium chloride.

It is difficult to determine whether these presumably enzyme activities take place within the bodies of the bacilli, on the surface or in the surrounding medium. There is much evidence in favor of the view that absorption of the substance to be acted upon is a preliminary step and that the products liberated from the bacteria become available to the other bacteria present. There is eventually acid production from these disaccharides by both types of microbe. Whether the final breakdown to gas occurs from the acids, just elaborated by the gas producer, or by the acid producer, it seems impossible to determine. It would appear from the evidence that the enzymes responsible for acid and for gas are different and that the acid-producing enzyme acts first; indeed this preliminary acidity may be necessary before the gas-producing enzyme can act.

The study of these phenomena opens up many problems concerning the finer metabolism of bacteria. The conditions favoring the activity of the bacterial transmissible lysin are those which stimulate bacterial growth and this phenomenon of autolysis requires physiologically active bacteria. Both have to do with the intimate physiological metabolism of the microorganisms, and the importance of knowing more about the surface changes of bacteria as these affect absorption and metabolism should be emphasized.

The two types of bacteria in the reactions we are considering have definitely different metabolic activities which are exhibited in acid production on one side and gas production on the other. That there are numerous other differences is self evident as suggested by the results of Speakman and Phillips [11] and many others. There is no question that all grades of the phenomena may obtain from no gas production by the combinations to various amounts of gas. These depend on many factors: the rates of growth of each of the pair; the time periods each reaches its maximum for its peculiar metabolic activity; the changes in the medium resulting from the growth requirement and metabolic activity of each; the interference or stimulation of various enzyme activities; the relative speed of these activities to each other; the effect on the cell membrane of these changes, as this alters absorption, and a great many other interrelationships. The two types of phenomena, inhibition of a function and the apparent manifestation of a new function are closely correlated.

The working hypothesis we have evolved is that the product resulting from the splitting of the test substance becomes available to both bacteria and the amount used by one or the other depends on the

functional activity of each at the particular time when this product is formed. Many factors alter this functional activity and on these depend our results. What this product is we are not prepared to say but we believe it may be different at different stages of the process. Monosaccharides are probably formed in the first stage; these are almost immediately transformed to various organic acids which in their turn are changed to carbonates or to gas. Which transformation occurs is dependent on the factors already mentioned.

CONCLUSIONS

Synergism is a useful term for the cooperative phenomena discussed in this paper.

One of the pair of bacteria must be capable of splitting the test substance and forming acid. The other must be able to form gas from monosaccharides.

The stage of growth and functional activity of the bacteria play important rôles in the phenomena.

The hydrogen-ion concentration and its regulation by the bacterial products are important factors in obtaining various results.

Gas production can be inhibited as well as stimulated and the two phenomena are closely related through the various factors affecting the metabolism of bacteria.

All grades of these phenomena can be obtained, depending on the characters of the bacteria used, their age and the use of different mediums.

The gas analysis shows a different gas ratio for the pair from that obtained with the gas producer alone.

There is good evidence that the acid and gas producing functions are different in time and probably depend on different endo-enzymes.

There is a wide field for the biochemist and physicist to explore to broaden our meagre knowledge of the intimate metabolism of bacteria.

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STUDIES ON BOTULINUM TOXIN IN THE ALIMENTARY TRACT OF HOGS, RABBITS, GUINEA-PIGS AND MICE *

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An explanation for the great variations in the susceptibility of animals of different species to oral injections of botulinum toxin was sought along two lines: the possibility of the destruction or adsorption of the toxin by the intestinal contents, and the permeability of the intestine to the toxin.

Preliminary tests were made on the adsorption of the toxin by the cecal contents of guinea-pigs. Mixtures of toxin and cecal contents were incubated at 37 C. for one hour. Toxin was found in high titer at the end of this period. The initial addition of N/3000 HCl or Na_2HPO_4 caused no striking change in the titer of the toxin in the mixture. The P_H of the cecal contents and toxin mixture was 6.0; by the addition of HCl it was changed to 5.4; by addition of Na_2HPO_4 to 7.4.

A similar experiment was carried out with the contents of the small intestine of a guinea-pig without any evidence of adsorption of toxin. Although the experiments were few they did indicate that if adsorption takes place it is a slow process.

Since it has been shown that toxin can be demonstrated in the intestine after several hours [1] the factor of permeability seemed to warrant further study. Four types of animals were used, each of them varying in their susceptibility to botulinum toxin depending upon the method of administration. Type A botulinum toxin was used throughout these experiments. It was produced by growing the organisms in beef heart medium for 10 days under a vaseline seal. The MLD of the toxin for mice was determined each time it was used by intraperitoneal injections of one cc. quantities. The MLD was taken as the greatest dilution of toxin which would kill a mouse within 48 hours.

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[1] J. Infect. Dis., 1926, 38, p. 174.

EXPERIMENTS ON HOGS

Thom, Edmondson and Giltner[2] fed a six months old hog 30 cc. of toxin (Boise strain) with an MLD of 0.005 cc. for guinea-pigs. No symptoms resulted. Three weeks later the same animal was given an injection of 5 cc. of toxin with an MLD of 0.0001 subcutaneously. Three days later the only symptom noted was a very slight incoordination of the hind quarters. The animal otherwise appeared normal. One week later the hog succumbed with botulism.

Our own studies on the effect of massive doses of botulinum toxin fed to hogs were as follows.

A Poland China pig weighing 14 pounds (6.3 kilos) was fed 10 cc. of sterile botulinum toxin type A by means of a stomach tube. The toxin had been stored in the ice chest for 57 days and had an MLD of .0002 cc. This hog showed no symptoms and 11 days later was fed 10 cc. of a type B toxin which had an MLD of .001 cc. The animal was kept under observation for 30 days and remained normal.

Two hogs each weighing about 50 pounds were fed 100 cc. and 75 cc. of unfiltered toxin which had an MLD of .00001 cc. The animals showed no symptoms and were injected intraperitoneally with toxin 17 days later. One received 5 cc. of toxin and the other 50 cc. The hog receiving the larger amount showed marked prostration within 3 days with no apparent mouth or throat symptoms. The abdomen was very much distended and the animal died on the 4th day. No characteristic lesions were noted at necropsy. The pig receiving the smaller dose of toxin remained normal.

A pig weighing about 50 pounds was injected with one cc. of a 1-10 dilution of toxin which had an MLD of .001 cc. for mice. A sample of blood was taken from the heart 90 minutes after the injection. One cc. of blood when injected into a mouse produced no symptoms. Four days later the pig was fed 90 cc. of unfiltered toxin with an MLD of .00001 cc. No symptoms developed in 14 days. A sample of blood taken 90 minutes after feeding failed to produce any symptoms in a mouse.

There is no doubt that hogs are very resistant to the toxin of Cl. botulinum type A given orally. As may be seen from the experiments, some hogs received with impunity 9 to 10 million MLD for mice. Considering the body weight, gram for gram, if hogs and mice were of equal susceptibility to intraperitoneal injections of toxin, then enough toxin was fed to one hog to kill from 6 to 7 thousand hogs. No toxin could be demonstrated in the blood of the hog which received 90 cc. of toxin or the equivalent of 9,000,000 MLD for mice. If this animal's blood volume were calculated as 1/14th of the body weight, there should be about 1600 cc. of blood. Providing there was no enhancement of toxin when mixed with serum and no binding or destruction of the toxin by the organs or tissues, if 0.016 of a cc. of the toxin entered the blood stream it could be detected by mouse injection. Not enough data are available regarding the susceptibility of the hog to other modes of injection of the toxin.

[2] J. Am. M. A., 1919, 73, p. 967.

EXPERIMENTS ON RABBITS

Van Ermengem[3] found that rabbits were quite resistant to botulinum toxin when it was fed, tolerating from 5 to 10 cc. When the same toxin was given by other routes rabbits were quite susceptible.

We have found great variation among rabbits fed toxin by means of a stomach tube. In a preliminary experiment an animal receiving 5 cc. of toxin died 2 days later than one receiving 2 cc. of the same toxin. In the same experiment an animal receiving 1 cc. died in 2 days.

Two rabbits weighing 1625 and 1855 grams respectively were each fed 10 cc. of toxin which had an MLD of .0001 cc. and had stood 5 days in the ice chest after filtering. One of the animals showed marked symptoms of botulism 3 days later and was found dead the following day. One-fourth cc. of serum secured after death failed to produce symptoms when injected into mice. The other rabbit remained normal and was observed over a period of three weeks.

TABLE 1

INJECTIONS INTO MICE OF BLOOD TAKEN FROM RABBITS RECEIVING INTRAVENOUS INJECTIONS OF BOTULINUM TOXIN

Rabbit	Weight, Gm.	Amount of Toxin Injected, Cc.	Calculated MLD per Cc. Blood	Time of Bleeding	Toxin in Blood in Dilution of 0.5 Cc. Injected: 0	1:10	1:100	Remarks
1	2,510	0.19	10.0	5 minutes	+	+	..	Rabbit showed marked symptoms in 6 hours. Died few hours later
				90 minutes	+	+	..	
				6 hours	+	s	..	
2	2,540	1.8	100.0	5 minutes	+	+	+	Rabbit died in 6 to 7 hours
				90 minutes	+	+	+	
3	3,650	0.013	0.5	5 minutes	+	..	..	Rabbit died in 2 days. 1 cc. of blood injected in mouse at 18.5 hour interval
				90 minutes	s	..	..	
				18.5 hours	+	..	..	
4	2,862	0.04	2.0	5 minutes	+	..	..	Rabbit died in 22 hours
				90 minutes	+	..	..	
				18.5 hours	+	..	..	
5	1,550	0.005	0.05	90 minutes	0	..	..	Rabbit lived
6	2,300	0.004	0.025	90 minutes	0	..	..	Rabbit lived

s = symptoms of botulism.

Two rabbits weighing 1850 and 2250 grams were fed 1 cc. and 10 cc. of toxin respectively (MLD .0001 cc.). One-half cc. portions of the blood from the first animal was taken from the heart 90 minutes after feeding, and injected into mice. The mice remained normal. The animal died a few minutes later apparently from shock and not from botulism. One-half cc. samples of blood were taken from the other rabbit (which had received 10 cc. of toxin) 90 minutes, and 6.5 hours after feeding, and injected into mice. The mice did not show any symptoms. The rabbit died 11 days later from an infection and not from botulism.

Small quantities of toxin were injected intravenously into rabbits, and cardiac punctures made at intervals. The blood samples so obtained were tested for toxin by mouse injection. The MLD of the toxin was .0001, with the exception of that injected into rabbits 1 and 5 which had an MLD of .001. The number of MLDs for mice per cc. of blood was calculated for a total volume equal to 1/14th of the weight of the animal. The injections were made into the ear veins and the animals were bled from the heart. The results are shown in table 1.

[3] Arch. de Pharmacodynamie, 1897, 3, p. 499.

In most cases one-half cc. quantities of the diluted blood were injected into mice. In the case of the 18.5 hour sample taken from rabbit 3 and of the samples taken at the 90-minute interval from rabbits 1 and 5, 1 cc. of blood was injected. In many instances the titer of the toxin in the blood stream was not obtained as the dilutions were not high enough.

Of special interest is the case of rabbit 3 which was given one-half of an MLD of toxin per cc. of blood. Five minutes after injection there were actually found more than 2 MLD, at the 90-minute interval less than 2, and at the 18.5-hour interval one MLD per cc. of blood. Perhaps this increased toxicity for mice may be due to the effect of the rabbit blood on the mice rendering them more susceptible to the toxin which the blood contained.

Injection of Toxin into the Small Intestine.—The animals were anesthetized with ether and an incision was made about 4 cm. in length in the mesogastric region. Five cc. of toxin with an MLD of .0001 were injected into the small intestine of each of two rabbits. The syringe used in making the injection was filled with toxin and then a sterilized 27 gauge needle was inserted in place of the one which might have some toxin on it. The point of injection was touched with tincture of ferric chloride. A short piece of silk thread was tied in the mesentery to mark the point of injection. One-half cc. portions of the heart blood taken 90 minutes after the injection of toxin were inoculated into mice. One cc. samples were taken after 18.5 hours. None of the mice showed symptoms of botulism. After 7 days both rabbits showed marked respiratory symptoms and one died a few hours later. The other rabbit was bled from the heart and one cc. of the blood injected into a mouse. The mouse was kept under observation for 5 days and remained well. This rabbit died 14 days after the injection, the symptoms of botulism persisting from the time when first noticed (7 days).

The liver of the rabbit which died in 7 days was very much congested. The spleen was much smaller than in the normal rabbit. The other organs appeared normal. The only way of determining the point of injection of the toxin was to locate the silk thread in the mesentery as the intestines showed no abnormalities. The silk ligature was free from adhesions and was located 6 feet 6 inches from the pylorus of the stomach and 4 feet 5 inches from the ileocecal valve.

The rabbit which died in 14 days was necropsied and the findings were identical to those described above. This rabbit gave birth to 6 full term young just before death. The urinary bladder was distended with urine. The stomach and intestines contained very little food material. A sample of blood was taken from the portal vein and centrifuged. Three-fourths of a cc. were injected into a mouse without producing symptoms of botulism.

The intestine at the point of injection appeared normal although there was some darkening in this area which may have been caused by the tincture of ferric chloride. The point of injection was 2 feet and 4 inches from the pylorus of the stomach and 8 feet and 3 inches from the ileocecal valve.

In two rabbits which received injections of toxin in the small intestine toxin could not be demonstrated in 0.5 cc. samples of blood taken after 90 minutes and in 1 cc. quantities taken after 18 hours. No toxin was demonstrated in one cc. of blood taken from one of the rabbits on the seventh day.

It is obvious from this experiment that no large quantities of toxin entered the blood stream although enough came through to produce death in both cases. Either the amount of toxin which was taken up by the blood stream was very small and acted over a long period of time or the toxin was carried by some other route.

Intracecal Injections of Toxin.—Two rabbits were given injections of toxin into the cecum. The technic of injection used was similar to that previously described. A small incision about 3-5 cm. in length was made along the linea alba in the mesogastric region. The cecum was injected in the larger portion. One rabbit weighing 3600 grams received 5 cc. of toxin and the other weighing 2090 grams, 0.5 cc.

The rabbit receiving the largest injection of toxin died the following day. One half cc. of blood taken from this animal killed a mouse in 16 hours. One-twentieth of a cc. produced marked symptoms of botulism but failed to kill a mouse. A necropsy was made on this animal with the following results: There was no peritonitis, but a small area on the cecum about 2 cm. in diameter was badly congested, probably at the point of injection. The wall of the cecum was ruptured 4.5 cm. from this point. The reason for the rupture was not known, but may have been caused by an error in technic either in making the injection or in handling the cecum during the operation. The animal undoubtedly died from botulism.

The rabbit which received 0.5 cc. of toxin remained normal.

EXPERIMENTS ON GUINEA-PIGS

Our perfusion experiments on rabbits and hogs showed that the toxin might be taken up by the blood stream in small amounts, in some cases within one and one-half hours. These results seemed to warrant a search for toxin in the blood of a more susceptible animal which had been injected with toxin in various portions of the alimentary tract.

Six guinea-pigs were each injected with 2.5 cc. of toxin with an MLD of .0002. Injections were made into the stomach of two, and the pyloric end ligated. Two were injected with toxin in or near the duodenum. Ligatures were placed about the pyloric end of the stomach and near the ileocecal valve in order to confine the toxin to the small intestine. Two received injections of toxin in the cecum and the ileocecal valve ligated. The technic of injection has been described previously in this paper. Cardiac punctures were made after 1.5 hours and the whole blood so obtained injected into mice. The guinea-pigs with one exception died from 6 to 16 hours after the injections were made. They were necropsied the following day and small amounts of clotted blood obtained from the heart were centrifuged and the serum injected into mice. Antitoxin control mice lived. The results of this experiment are given in table 2.

Two guinea pigs were given intracecal injections of one cc. of botulinum toxin which had an MLD of .001. The small intestine near the ileocecal valve was not ligated as in the previous cases. One of the animals had an infection at the site of operation which rapidly healed. This animal died on the 17th day with symptoms of botulism.

The other guinea-pig receiving the same dose remained normal. On the 17th day this animal was fed 1 cc. of the same toxin. Four days later definite symptoms of botulism developed and the animal died on the 7th day.

In these experiments with one exception, which may have been due to an error in technic, toxin was not found in the heart blood 90 minutes after it was injected. Toxin was demonstrated in the heart blood taken

TABLE 2

Injections into Mice of Blood Taken from Guinea-Pigs Which Had Received Gastro-Intestinal Injections of Botulinum Toxin

Guinea-Pig	Site of Injection	Time of Bleeding, Hours	Amount Injected, Cc.	Toxin	Remarks
1	Stomach.....	1.5 6 to 16	1.0 0.45	0 s	Stomach wall necrotic along the greater curvature
2	Stomach.....	1.5 6 to 16	1.0 0.45	0 +	Stomach wall necrotic along the greater curvature
3	Small intestine.....	1.5 6 to 16	1.0 0.17	0 0	
4	Small intestine.....	1.5 6	1.0 1.0	0 0	Small intestine ruptured about 6 inches from ileocecal valve allowing escape of contents into body cavity. Died 5 hours
5	Cecum.....	1.5 6 to 16	1.0 0.32	s 0	Intestine had ruptured through wound
6	Cecum.....	1.5	1.0	0	Intestine had ruptured through wound. Cecum highly congested

s = symptoms of botulism.

TABLE 3

The Persistence of Botulinum Toxin in the Stomach and Small Intestine of Mice

Time Killed After Feeding Toxin, Hours	Mouse Inoculations for the Presence of Toxin	
	Toxin Present in 1 Cc. of a Dilution of	Toxin Not Present in
0.....	1:200	1:2000
0.....	1:200	1:2000
3.....	1:78	1:780
3.....	1:28	1:280
12.....	0	0.5 cc.
12.....	0	0.5 cc.
24.....	0	0.5 cc.
24.....	0	0.5 cc.

at necropsy from the two animals which received injections into the stomach. Necrosis and complete digestion of the stomach wall along the greater curvature occurred in both of these cases. This effect of botulinum toxin on the stomach has been referred to in another paper [4] and is mentioned by Van Ermengem.[3]

[4] J. Infect. Dis., 1925, 36, p. 383.

EXPERIMENTS ON MICE

Observations were made on the behavior of botulinum toxin in the alimentary tract of mice.

A series of 14 mice were each fed 0.5 cc. of toxin with an MLD of .001 by means of a silver sinus catheter. The mice were killed at intervals and the stomach and small intestines removed, ground in a mortar, diluted with sterile saline solution and centrifuged for one hour at high speed. The supernatant fluid was titrated for toxicity.

Antitoxin was given the mice which received the lowest dilution of the supernatant fluid from the macerated viscera. None of them died. Six mice which were fed 0.5 cc. of toxin were kept as controls and remained alive and well. The protocol of the experiment is given in table 3.

SUMMARY

There was no evidence that type A botulinum toxin was adsorbed by the intestinal contents in vitro even where the P_H was shifted with N/3000 HCl or Na_2HPO_4.

Hogs were found to be very resistant to large oral doses of toxin; in some cases as much as ten million MLDs for mice were fed without producing any ill effects. Toxin was not demonstrated in one cc. of blood taken from a hog which had been fed nine million (mouse) MLDs.

Toxin was not demonstrated in the blood of rabbits 90 minutes after feeding botulinum toxin.

Two rabbits received intravenous injections of small graded doses of toxin. Toxin was demonstrated in the blood stream 18 hours later.

Two rabbits receiving large doses of toxin in the small intestine showed symptoms of botulism within seven days. One died on the 7th day and the other on the 14th day. Toxin was not demonstrated in one-half cc. of blood taken from each of these animals after 90 minutes or in one cc. taken after 18 hours.

Two rabbits received intracecal injections of toxin. One of the rabbits remained normal. The other died the following day and toxin was found in the blood.

Toxin could not be demonstrated after 90 minutes in the blood of guinea-pigs which had received injections of toxin in the ligated stomach but was demonstrated in the serum from the heart blood taken after death. Toxin was not demonstrated in the blood of animals receiving injections of toxin in the ligated small intestines or ligated cecum, either after 90 minutes or in the serum from the heart blood taken after death. There was one exception in a guinea-pig which

had received an intracecal injection, the cecum of this animal being ruptured.

Two guinea-pigs were given intracecal injections of toxin; one died 17 days later with typical symptoms of botulism; the other remained normal.

Mice fed botulinum toxin failed to develop any symptoms of botulism. Toxin was demonstrated in the stomach and small intestines of mice 3 hours after they had been fed toxin but not 12 hours later.

The evidence obtained from these experiments indicates that, if an animal susceptible to oral administration of toxin has been fed toxin, in addition to the toxin absorbed from the stomach and small intestines there is perhaps some continued absorption from the cecum, although the amount is probably very slight. Further studies are being made on the absorption of toxin from the cecum.

PERMEABILITY OF THE SMALL INTESTINE OF RABBITS AND HOGS TO BOTULINUM TOXIN*

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The object of these experiments was to determine any difference in the permeability of the small intestine of animals resistant and susceptible to botulinum toxin. The method of perfusion with blood from the same animal was chosen to eliminate the effect which organs and other tissues of the body might have on the toxin.

Type A toxin was used throughout the experiments. It was produced by growing Cl. botulinum in beef heart medium for 10 days under a vaseline seal. The toxin was then passed through a Berkefeld filter and sealed off in 10 cc. quantities in test tubes. The titer of the toxin was determined by intraperitoneal injection in mice each time it was used.

The hog, which is very resistant to large oral doses of the toxin, and the rabbit, which is comparatively susceptible, were used. Although the guinea pig would have been preferred to the rabbit, since it is still more susceptible to oral injections of the toxin, it was found too small for this work.

The animals were anesthetized with ether and the carotid arteries cannulated. The blood was prevented from clotting either by the addition of heparin or by defibrinating. In some of the later experiments the animals were injected with about 0.1 gram of heparin in 0.85% salt solution before anesthetizing. Blood from these animals did not clot for several hours.

In rabbits the main trunk of the superior mesenteric vein and artery were cannulated while in hogs branches of these vessels were cannulated. In some of the earlier experiments the blood was diluted with Ringer-Locke solution but as this mixture was found to produce edema its use was discontinued.

The blood coming from the vein was oxygenated and pumped by means of a Woodyatt pump [1] through a coil of tubing in a water bath,

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[1] J. Biol. Chem., 1917, 29, p. 355.

kept at between 38 and 40 C., into the arterial circulation. The loop of intestine was carefully ligated and packed in gauze saturated with warm salt solution. Five cc. of toxin, unless otherwise specified, were injected into that portion of the loop nearest the duodenum. The point of injection was touched with tincture of ferric chloride and tied off. The loop was carefully manipulated so as to distribute the toxin throughout its length. In all cases marked peristalsis was present throughout the experiment. Such movement would serve to mix the contents much more thoroughly than the manipulation given at the beginning of the experiment.

Samples of blood were taken at definite intervals from the venous circulation. In some cases the whole blood was titrated for the presence of toxin while in other instances the serum was used. The details are given in the experiments.

Exper. 1.—Rabbit, 4,000 grams. The carotid arteries were cannulated and the blood collected, defibrinated, filtered and diluted with equal quantities of Ringer-Locke solution. A loop of intestine 2 feet long, 4.5 feet from the pylorus of the stomach, and 1.5 feet from the ileocecal valve was perfused first with Ringer-Locke solution which was immediately replaced with the diluted blood.

Perfusion was maintained 30 minutes from the time the toxin was introduced into the intestine. A mouse which received 0.5 c c. of serum from a sample of venous blood taken at the end of the perfusion showed symptoms of botulism but recovered. Another mouse injected with 0.5 c c. of oxygenated blood showed no symptoms. The toxin used had an MLD of 1-10,000 for mice. At the end of the experiment some blood was present in the intestine about two or three inches from the ligature nearest the stomach. There was a little edema, the loop being distended with fluid.

Exper. 2.—Rabbit, 3,500 grams. The blood was defibrinated, filtered and diluted three to four times with Ringer-Locke solution. The abdominal aorta was cannulated and the intestine perfused with Ringer-Locke solution before a loop was ligated for perfusion. A loop of intestine 5 feet in length, 3.5 feet from the pylorus of the stomach and one foot from the ileocecal valve was perfused with diluted blood. In this case 6 c c. of toxin was used and injected into the loop at the beginning of the perfusion.

The intestine was perfused 1.5 hours. One half c c. of serum from a sample of blood taken at the end of the experiment killed a mouse in one day, while the same quantity of a 1-10 dilution was not toxic. Antitoxin control mice remained well. The toxin used had an MLD of 1-10,000 for mice.

At the end of the experiment there was considerable edema, the loop was distended with bloody fluid and parts of the intestine were quite hemorrhagic.

Exper. 3.—Rabbit, 4,200 grams, was injected intravenously with heparin about 10 minutes before bleeding. Ringer-Locke solution was not used in this experiment. A loop of intestine 6 feet in length, 3 feet from the pylorus of the stomach and 1.5 feet from the ileocecal valve was perfused with the heparinized blood for 1 hour and 5 minutes.

The toxin used had an MLD of 1-10,000 for mice. A half c c. of blood taken at the end of the experiment killed a mouse in one day. A mouse

receiving 0.5 c c. of serum taken at the same time showed symptoms but recovered. About ⅓ of the centrifugalized blood was serum and ⅔ were cells.

At the end of the experiment the loop of intestine was somewhat discolored, of a dark red color and contained 100 c c. of contents, a large portion of which was blood. A mouse which received 0.5 c c. of a 1-100 dilution of the supernatant from the centrifuged contents died in one day. A dilution of 1-1,000 produced no symptoms. Antitoxin controls all remained well. The calculated MLD of the loop contents was 0.5 c c. of a 1-250 dilution.

Exper. 4.—Rabbit, 4,000 grams. The blood was defibrinated, filtered and diluted from 60 c c. to 240 c c. with Ringer-Locke solution. About 115 c c. of diluted blood was lost during the perfusion. The intestines were first perfused with Ringer-Locke solution injected into the abdominal aorta. A loop of intestine 2.5 feet from the pylorus of the stomach, 4.5 feet long and one foot from the ileocecal valve was perfused with the diluted blood, for 1.75 hours, after the toxin was introduced. The intestine showed marked peristalsis after 1.5 hours of perfusion but not 15 minutes later. The loop was distended with fluid and was hemorrhagic especially in the upper 4 inches after 1.5 hours. The toxin had an MLD of 1-10,000 for mice. Toxin was not demonstrated in 0.5 c c. quantities of blood taken at 1.5 or 1.75 hours.

Exper. 5.—Rabbit, 3,800 grams, was injected with heparin solution shortly before bleeding. A second rabbit was also injected with heparin in order to provide enough blood for longer perfusion. Ringer-Locke solution was not used in this experiment. About 50 c c. of blood was lost during the perfusion from the pump and in the oxygenation chamber. Blood centrifugalized after perfusion was about ⅓ serum and ⅔ cells.

The loop of intestine perfused was about 6 feet long, 2.5 feet from the pylorus of the stomach and 1.5 feet from the ileocecal valve. Perfusion was continued for 1.25 hours. At the end of this time the intestine was congested and of a dark red color containing 60 c c. of very bloody material.

The toxin had an MLD of 1-10,000 for mice. Toxin was not demonstrated in either the serum or the whole blood taken at the end of the experiment. The mouse which received 0.5 c c. of a 1-100 dilution of the supernatant from the centrifuged intestinal contents died in 24 hours. The mice receiving 0.5 c c. of the 1-500 and 1-1,000 dilutions showed very marked symptoms. All antitoxin control mice remained well. The calculated MLD for the loop contents was 0.5 c c. of a 1-100 dilution.

Exper. 6.—Rabbit, 3,850 grams, was given approximately 0.1 gm. of heparin in 0.85% salt solution just before bleeding. The carotid arteries were cannulated and 100 c c. of blood obtained. Ringer-Locke solution was not used in this experiment. Seven c c. of blood was lost during two hours and twenty minutes of perfusion.

A loop of intestine 5 feet from the pylorus of the stomach, 5.5 feet in length and 1.5 feet from the ileocecal valve was perfused. After 2 hours of perfusion the intestine appeared normal, showing marked peristalsis and no edema. Twenty minutes later there were a few scattered red spots on the intestinal wall and at this time it was noted that the blood had started to clot. Centrifugalized blood taken at the 2 hour interval was about half cells and half serum. There were 55 c c. of loop contents, yellowish in color and normal in appearance with no trace of blood.

The toxin used in this experiment had an MLD of 1-10,000. The mice recceiving half c c. quantities of blood and serum taken at the 1.5-, 2-, and 2.3-hour intervals showed marked symptoms of botulism. A mouse receiving 0.5 c c.

of the whole blood taken at the 1.5 hour interval died in 4 days. A mouse receiving 1 c c. of serum from the 2 hour sample died in 2 days. Antitoxin control mice remained well.

A titration for the toxicity of the loop contents showed that 0.5 c c. of the supernatant fluid from the centrifuged material killed a mouse in a 1-100 dilution in 2 days, the mouse receiving the 1-500 dilution showed symptoms while that receiving the 1-1,000 dilution remained well. Antitoxin control mice remained well in all cases. The calculated MLD for the loop contents was 0.5 c c. of a 1-450 dilution.

Exper. 7.—A hog weighing about 25 kilos was given an intravenous injection of heparin solution. The heparin was found to be insufficient to prevent clotting and the blood was then defibrinated.

A loop of intestine 3.7 feet in length, 11 feet from the pylorus of the stomach and 27 feet from the ileocecal valve was perfused first with 0.85% salt solution and then with defibrinated blood. Perfusion was maintained for 1.5 hours after 10 c c. of toxin was injected. The preliminary perfusion with salt solution caused slight edema and some flow of liquid into the intestine.

The toxin used in the experiment had an MLD of 1-10,000. More than half the volume of a sample of blood taken at the end of the experiment and centrifuged was serum. One half c c. of serum taken at a one hour interval produced marked symptoms in a mouse. One half c c. of whole blood taken after 1.5 hours of perfusion produced very marked symptoms while 0.5 c c. of serum taken at the same time killed a mouse in 4 days. Antitoxin control mice remained well.

There were about 90 c c. of intestinal contents which were very fluid and of a light pink color. A mouse receiving 0.5 c c. of a 1-100 dilution of the supernatant from the centrifugated intestinal contents died in one day, a mouse receiving 0.5 c c. of a 1-1,000 dilution died in 4 days while the 1-5,000 remained well. The MLD calculated for the intestinal contents is 0.5 c c. of a 1-500 dilution.

Exper. 8.—A hog weighing about 22 kilos was used. The blood was defibrinated. A loop of intestine 4 feet long, 1.5 feet from the pylorus and 30 feet from the ileocecal valve was perfused first with a little blood to which had been added about 0.15 gram of heparin. Ringer-Locke solution was not used in this experiment.

About 100 c c. of blood were placed in the apparatus and more added after the 1.5 hour interval. Perfusion was continued for 2 hours. About two thirds of a sample of blood, taken after perfusion and centrifugalized, was cells. Toxin was not demonstrated in the serum or whole blood in any sample.

Some of the return vessels from the loop contained small emboli made up of blood clots which by no means occluded the circulation. About 100 c c. of contents was found in the loop which were slightly colored by blood. The toxin injected into the loop had an MLD of 1-10,000. A mouse which received 0.5 c c. of a 1-100 dilution of the supernatant from the centrifuged intestinal contents died in 2 days. The mouse receiving 0.5 c c. of a 1-500 dilution showed symptoms while the 1-1,000 remained normal. The MLD of the intestinal contents was calculated as 0.5 c c. of 1-250 dilution.

DISCUSSION

In five out of eight experiments botulinum toxin was demonstrated in the venous blood from a perfused loop of intestine containing toxin. Only a very small amount of toxin was demonstrated, often enough

only to produce symptoms in a mouse when 0.5 cc. quantities were given. Toxin was demonstrated in the venous blood from a perfused loop of intestine of one hog but not from the other.

The experiments agree quite closely and it may be that toxin was present in the blood from the intestine in every case, but that the amount was too small to detect. Special attention may be called to experiment 6 in which the intestine appeared normal after 2 hours of perfusion. In this experiment no more toxin was demonstrated after 2 hours and 20 minutes than after 1.5 hours of perfusion.

There was of course some dilution of the toxin with the blood in the perfusion system but a much greater dilution would take place in a complete normal circulatory system. It must also be remembered that the addition of protein to a toxin may enhance the toxicity so that even smaller amounts of toxin may be detected.[2]

The amount of toxin in the total blood volume of the perfusion system was perhaps in no case sufficient to kill the animal. There was enough toxin placed in the loop of intestine to kill hundreds of rabbits [3] if given intravenously. The results of these experiments are consistent with what is actually found when toxin is fed. Toxin in the loop of intestine diminished very little in potency during the period of the experiment.

SUMMARY

A loop of small intestine in each of six rabbits was injected with botulinum toxin and perfused with blood from the same animal for intervals varying from 30 minutes to 2 hours and 20 minutes. Very small quantities of toxin were demonstrated in the perfused blood, often only symptoms of botulism were produced in the mice receiving 0.5 cc. quantities of the undiluted blood or serum. Never was any toxin demonstrated in the 1-10 dilution.

Two hogs were treated in a similar manner with the result that toxin was demonstrated in one case and not in the other. Only a very small amount of toxin was found in the one case, a mouse receiving 0.5 cc. of serum taken at an hour and a half interval died in 4 days.

The toxin introduced into the ligated loop of intestine showed little if any decrease in potency during the course of the experiment.

[2] Jensen, L. B.: Reactions of Heterologous Nonspecific and Normal Serums on Botulinum Intoxication. (In press.)

[3] Dack, G. M., and Gibbard, J.: J. Infect. Dis., 1926, 39, p. 173.

BACTERIOLOGIC STUDIES IN ENDOCARDITIS

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It was the purpose of this study to search for distinctive features in the group of streptococci associated with streptococcus endocarditis. The criteria adopted were morphological, cultural and immunologic characteristics; animal experimentation and toxin formation. Incidentally, the presence of antibodies in the blood of patients suffering from the disease was investigated.

The recognition of a distinct form of endocarditis, has been of comparatively recent date and of slow evolution. Although of only moderately common occurrence, the disease is extremely important, since most of the cases studied have been fatal.

Hemsted [1] in 1855 first suggested an infectious origin for the endocarditis associated with uterine infections, and attention was thereafter directed to the finding of bacteria in the vegetations in both ulcerative and verrucous endocarditis. Heiberg [2] described a case of acute endocarditis which had been reported by Winge in 1869 in which micrococci were found in the vegetations and added another case of acute ulcerative endocarditis in which small micrococci were present in the vegetations.

For a short period it was thought that bacteria were present in all forms of endocarditis. Thus, Klebs [3] felt that he could separate rheumatic from septic cases by the character of the cocci found in microscopic sections. Koster [4] thought that verrucous endocarditis was a chronic and healed stage of ulcerative endocarditis and hence originally of bacterial origin. It may be noted that this idea of healed bacteria-free endocarditis has been supported with new evidence in recent years by Libman [5] but with the distinction that the healed cases are supposed to have been of the subacute bacterial type.

Later investigators failed to demonstrate bacteria in the lesions of verrucous endocarditis. Hamburger [6] examined ten such cases with

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[1] Lancet, 1913, 1, p. 10.
[2] Virchows Arch. f. path. Anat., 1872, 56, p. 407.
[3] Arch. f. exper. Path. u. Pharmakol., 1878, 9, p. 52.
[4] Virchows Arch. f. path. Anat., 1878, 72, p. 257.
[5] Am. Jour. Med. Sc., 1913, 146, 525.
[6] Ueber akute Endocarditis in ihrer Beziehung zu Bacterien, Berlin, 1879.

negative results and Weichselbaum [7] questioned whether verrucous endocarditis was due to microbic invasion. This view received further support from Wyssokowitch [8] and Orth.[9]

Additional interest was lent to the discussion by the experimental production of acute endocarditis by previously puncturing the aortic leaflets according to a method devised by Rosenbach.[10] Wyssokowitch [8] was able to infect the injured valve by injecting pyogenic streptococci. Ribbert [11] produced acute endocarditis merely by injecting a suspension of staphylococci and fine particles of potato intravenously. Many reports of the production of endocarditis by injecting cultures of streptococci have been subsequently published.

As the facts then stood, there were two general types of endocarditis, namely, those associated with bacteria and those in which no direct connection with bacteria could be proved. The introduction of blood culture methods was responsible for separating the bacterial group into acute and chronic cases. Of the early reports, that of Kühnaw [12] is noteworthy, in that 66 cases of rheumatism, many of which were accompanied by endocarditis, gave negative cultures, the finding of sarcinae, staphylococci and Bacillus subtilis being discounted.

It soon became evident that among those cases of endocarditis which gave definitely positive blood cultures a certain group could be distinguished because of their subacute or chronic character. These cases most frequently yielded streptococci from blood cultures. This was noted by Harbitz.[13] Later Horder [14] very accurately described cases of acute and chronic infectious endocarditis of which 17 were subacute and chronic and yielded blood cultures of "saprophytic streptococci." In the following year Schottmüller [15] individualized this type of endocarditis with "endocarditis lenta," a disease of slow, insidious onset, definite bacteriology and symptoms of injured heart valves with bacteremia. The five cases which he described were caused by streptococci of the green-producing variety. In the same year Libman and Celler [16] described the same disease under the name of "subacute bacterial" endocarditis and found later [17] that while most of the streptococci

[7] Centralbl. f Bakteriol., I, O., 1887, 2, p. 209.
[8] Virchows Arch. f. path. Anat., 1886, 103, p. 301.
[9] Ibid., p. 333.
[10] Arch. f. exper. Path. u. Pharmakol., 1878, 9, p. 1.
[11] Deutsch. med. Wchnschr., 1885, 11, p. 717.
[12] Ztschr. f. Hyg. u. Infektionskrankh., 1897, 25, p. 492.
[13] Deutsch. med. Wchnschr., 1899, 25, p. 121.
[14] Quart. J. Med., 1908-1909, 2, p. 289.
[15] München. med. Wchnschr., 1910, 57, p. 617.
[16] Am. J. M. Sc., 1910, 140, p. 516.
[17] Ibid., 1912, 144, p. 313.

produced green on blood agar plates, some were indifferent and some produced slight clearing. They also stated that the streptococci found were not very virulent.

While the bacteria obtained are almost always streptococci, other organisms, such as influenza bacilli, pneumococci and gram-negative cocci have been described.[18] The cases on which the present study was based were all cases of streptococcus infection. The characteristics of these streptococci have been studied by others, and such features as low virulence and their effect on blood agar have been described. Horder [14] studied the fermentation reactions of the streptococci from seventeen such cases from the standpoint of the grouping proposed by Andrews and Horder [19] and found that nine belonged to the salivarius type and eight to the fecalis type. Major [20] studied the complement-fixing antibodies in the serum of a patient and concluded that the reaction was not specific, since it was also obtained with an antigen made from Streptococcus pyogenes. Rosenow [21] claims that the bacteria usually isolated from these cases are "modified pneumococci," and tries to show that "the continuation of infection and death are largely due to a process of bacterial immunization against the antibodies of the host rather than to the virulence in the usual sense of the infecting bacteria."

The fourteen cases herein described were typical of the disease. A brief synopsis of each case follows:

Case 1.—Colored, aged 19, single, came into the hospital complaining of shortness of breath, sharp pains over the precordia, and aching over body, particularly in the joints. She had chicken pox, whooping cough, tonsillitis frequently, influenza five years ago, and rheumatic fever at 15 years of age. She also had dropsy at this time and frequently spat blood. Nocturia for the past eight months and polyuria (4-5 times during day). She weighed 145 pounds and had fluctuating fever while in the hospital (3 months). There was a diastolic murmur over aortic valve area and systolic murmur at the apex. The joints were very tender. Streptococcus viridans was isolated from blood culture and vegetations on the mitral valve. The Wassermann test was negative, blood urea 32, uric acid 7, sugar 95. Clinical diagnosis: acute endocarditis, aortic and mitral insufficiency, cardiac hypertrophy and decompensation.

Case 2.—Colored laborer, entered the hospital complaining of pains in chest, had slight fever, cough and expectoration. Previously he had measles, whooping cough, mumps. He was emaciated, had râles over bases of both lungs, loud systolic murmurs and pericardial friction rub. The heart appeared strong and overacting. The clinical diagnosis was subacute endocarditis and lobar pneumonia. Pneumococcus group 4 was isolated from the sputum and Streptococcus viridans from the blood. The Wassermann test was negative. The urine contained a trace of albumin and granular casts. He left the hospital at the end of a month greatly relieved.

Case 3.—Negress, 45 years of age. Her chief complaint was shortness of breath and swelling of feet. She had smallpox, chicken pox, measles, mumps and whooping cough, typhoid fever two years previously and malaria during the past year. She had two children and two miscarriages. The present trouble began a year ago when patient had her menopause. She had hot flushes, air hunger, hot feet and palpitation of heart. Five months ago she had an attack of indigestion and vomited a great deal of fluid. Since then she has been nervous and unable to sleep soundly. Her tonsils were very much enlarged. Smal lamount of fluid was drained from

[18] Simons, I.: Quart. J. Med., 1913-1914, 7, p. 291.
[19] Lancet, 1906, 2, p. 775.
[20] Bull. Johns Hopkins Hosp., 1912, 23, p. 326.
[21] J. Infect. Dis., 1909, 6, p. 245; 1910, 7, p. 429.

the peritoneal cavity and she had a draining sinus. Three days before death she became stuporous and it was impossible to clear up her edema. Streptococcus viridans was isolated from the blood. Wassermann test was negative and blood chemistry was normal. Clinical diagnosis was subacute endocarditis and chronic nephritis.

CASE 4.—Patient, aged 29, complained of pain in joints (knees and ankles). He had a systolic murmur. Wassermann and colloidal gold tests were negative. The urine showed a heavy cloud of albumin and hyalin casts. Blood urea was 40, uric acid 3.8. Unable to get early history. At necropsy vegetations were found on the mitral valve. Streptococcus viridans was isolated from the vegetations and from a splenic infarct. The clinical diagnosis was subacute endocarditis and acute nephritis.

CASE 5.—White laborer, aged 42. For past year patient complained of pains in chest and shortness of breath. He had had common childhood diseases. His tonsils were enlarged and ragged. The Wassermann test was negative; blood chemistry normal; blood culture negative. At necropsy Streptococcus pyogenes was isolated from the vegetations on the mitral valve. This organism later lost its ability to hemolyze blood cells and became indifferent on blood agar. Clinical diagnosis was subacute endocarditis.

CASE 6.—Widower with no children, aged 58. Had pains in chest and ankles. Feet were constantly perspiring and temperature usually 99 F. He had typhoid and chancroid 18 years ago and pleurisy recently. Had an enlarged prostate gland and eye reaction to lues but denies chancre. He had a systolic heart murmur. Streptococcus viridans isolated from blood culture. The Wassermann test was negative; urine showed faint trace of albumin; sputum negative for tubercle bacilli. Diagnosis was subacute endocarditis.

CASE 7.—Patient, aged 81 years, was in the hospital only one week before she died. History is incomplete. She was emaciated and mentally abnormal. Blood culture at autopsy showed Streptococcus viridans. Clinical diagnosis was subacute endocarditis, general arteriosclerosis and senility.

CASE 8.—This strain was received from another hospital and history is incomplete. The clinical diagnosis was subacute endocarditis and pregnancy. Streptococcus viridans was isolated from the blood. Systolic murmur was quite marked.

CASE 9.—Colored laborer, aged 27, came into the hospital complaining of a swollen and painful ankle and shortness of breath (3-4 months). Had measles and malaria at 17 years of age. Has been married two years and has no children. Had gonorrhea four years ago, tonsillitis two years ago. Nocturia (4-5 times). He had a loud systolic murmur over apex of heart. Had moderate sclerosis. Denied lues. Temperature was irregular, almost septic. His Wassermann was four plus; had leukocytosis; large amount of albumin in urine; blood chemistry normal. Streptococcus viridans was isolated from blood culture twice and from vegetations on the mitral valve at necropsy. Clinical diagnosis was acute endocarditis and syphilis.

CASE 10.—Patient, 24 years of age. Had endocarditis ten years previously with long convalescence and recovery. As a member of the merchant marine, he led the roughest kind of life, and during this time contracted both syphilis and gonorrhea. He was admitted to the hospital with a septic temperature but apaprently not acutely ill. Two blood cultures showed Streptococcus viridans. He was discharged at the end of three months and is now apparently leading a normal life. Clinical diagnosis was subacute endocarditis and syphilis.

CASE 11.—This was an ambulatory case of chronic endocarditis. Streptococcus viridans was isolated from the blood.

CASE 12.—Colored patient, aged 23, came into the hospital talking at random and had pains in the head. Had chancre and gonorrhea nine years ago. Moist râles were heard in apex of right lung. The heart had a distinct diastolic murmur. Ran a continued elevated temperature. Corrigan pulse was pronounced. Wassermann test was negative; urine showed heavy trace of albumin and a few hyaline casts. Streptococcus viridans was isolated from the blood on two occasions and from the mitral valve vegetations at necropsy. Clinical diagnosis was subacute endocarditis and chronic nephritis.

CASE 13.—A 15 year old girl who died after three weeks in the hospital. She had acute rheumatic fever when 12 years old, was emaciated and teeth were in bad condition. Heart was very large and Streptococcus viridans was isolated from the blood. She had nocturia (4.5 times). Acute sore throat troubled her frequently. Clinical diagnosis was acute endocarditis and anemia.

CASE 14.—Colored man, 30 years of age, fairly well developed, had infected pharynx and fetid breath. Physical examination showed moist râles over upper lobe, apex of heart an inch outside of nipple line, systolic murmur. He complained of shortness of breath, a cold with pain in right chest and coughed up yellowish mucoid sputum. He never had an infectious disease. While in the hospital he developed acute arthritis, his left elbow becoming swollen, hot and tender. He had fever constantly. Urine analysis showed trace of albumin and few casts. Wassermann test was negative; sputum negative for tubercle bacilli. Streptococcus viridans was isolated from two blood cultures during life, and from the vegetations on the mitral valve and heart blood at autopsy. Clinical diagnosis was subacute endocarditis and lobar pneumonia.

CULTURE MEDIUMS

Beef Infusion Broth.—500 grams of finely chopped lean beef was placed in container with 1000 cc. of water and allowed to stand in the ice box overnight. The infusion was then boiled thirty minutes over an open flame, filtered through gauze and then through wet filter paper to remove the fat. Volume was brought up to 1000 cc. by the addition of water. To this infusion was added 10 gm. peptone (Difco), 5 gm. sodium chloride. Mixture was boiled over open flame until ingredients were dissolved and reaction adjusted to P_H 7.8., then filtered through filter paper and given fractional sterilization in the Arnold.

Brain Broth.—To 1000 cc. infusion broth was added 10 gm (1%) glucose. Long test tubes were three-quarters filled and several pieces of brain added. (Calf brain was cut into blocks about one cc. in size and washed in running water to remove blood.) These tubes were sterilized in the autoclave at twelve pounds for twenty minutes.

Trypsinized Broth.—To 1000 cc. sterile infusion broth was added 10 cc. of a 10% solution of trypsin in normal sodium chloride which had previously been sterilized by passing through a porcelain filter (Mandler 4).

CULTURE METHODS

Blood Culture.

Twenty cc. of blood was the usual amount taken for cultural purposes. Of this quantity:

3 cc. was placed in 75 cc. infusion broth with reaction of P_H 7.6.

1 cc. in tube of brain broth (P_H 7.6).

1 cc. in tube of trypsinized broth (P_H 7.6).

10 cc. was allowed to clot and the latter dropped in 50 cc. dextrose (0.2 %) beef infusion broth (P_H 7.6). This is a modification of Clawson's [22] method. The clot seems to provide partial anerobic conditions and any antibodies present are diluted in fluid medium. The serum separated was used for agglutination tests.

Concentration Method—To 5 cc. of blood was added 45 cc. sterile distilled water. This tube was then centrifugalized at high speed for ½ hour. After discarding the supernatant liquid, the residue was emulsified in 10 cc. infusion agar (P_H 7.6) melted and cooled to 45 C. and a plate poured.

These cultures were incubated at 37.5 C. The streptococci usually grew out in less than five days but no cultures were considered negative until they remained sterile at the end of one month's incubation.

Cultures at the Autopsy Table.

Heart Blood Cultures—Blood was collected with the utmost precautions direct from the heart chamber after searing the outside of the heart and and an opening through its wall. The blood was collected in a sterile pipette and placed in 75 cc. glucose broth. Subcultures were made from this broth on blood agar plates.

Infarcts of Spleen, etc.—The surface of the spleen over the infarct was seared and an opening made through the capsule with a hot knife. A stiff inoculating wire was inserted well beyond the depth of the stab puncture made by the hot knife and blood agar plates inoculated with the material collected.

[22] J. Infect. Dis., 1925, 36, p. 446.

Vegetations of the Heart Valve. —Forceps and scissors were sterilized in the flame and portions of the vegetations cut off and placed in a sterile petri dish. From this material blood agar plates and broth tubes were inoculated. Cultures were incubated at 37.5 C.

MORPHOLOGIC AND CULTURAL CHARACTERISTICS

Most workers who have isolated organisms from endocarditis patients report diplococci or streptococci. Poynton and Paine[23] called their organism a diplococcus. The action on the blood agar plate is not well described in the older literature. Beaton and Walker[24] and Walker and Ryffel[25] have described their strains as producing a brownish discoloration. Lyall[26] found his strains to be inactive on blood. Rosenow[27] reported strains some of which were inactive on blood,

TABLE 1

ORIGIN OF STRAINS OF STREPTOCOCCUS VIRIDANS

	Source of Culture	Clinical Diagnosis	Pathological Diagnosis
1	Blood	Acute endocarditis	Acute endocarditis; aortic and mitral insufficiency; cardiac hypertrophy and decompensation
2	Blood	Subacute endocarditis; lobar pneumonia	Subacute endocarditis; lobar pneumonia
3	Blood (at necropsy)	Subacute endocarditis; chronic nephritis	Subacute endocarditis; chronic nephritis
4	Splenic infarct	Acute endocarditis; acute nephritis	Acute endocarditis with septic infarcts in spleen, kidney and myocardium
5	Vegetations on mitral valve	Subacute endocarditis	Subacute endocarditis
6	Blood	Subacute endocarditis	
7	Blood (at necropsy)	Subacute endocarditis	Subacute endocarditis; cardiac decompensation
8	Blood	Subacute endocarditis; pregnancy	
9	Blood and vegetations on mitral valve	Acute endocarditis; syphilis	Acute endocarditis; syphilis
10	Blood	Subacute endocarditis	
11	Blood	Chronic endocarditis (ambulatory case)	
12	Blood and vegetations on mitral valve	Subacute endocarditis; chronic nephritis	Subacute endocarditis; chronic nephritis
13	Blood	Acute endocarditis; anemia	
14	Blood	Subacute endocarditis; lobar pneumonia	

some produced green, while others were hemolytic. Swift and Kinsella[28] found that their organisms belonged in the viridans group. They believe that the streptococci described by earlier workers correspond to what is now known as Streptococcus viridans.

The biologic tests, as reported in the literature, have shown that not all organisms isolated from endocarditis patients belong to the same group. Andrewes and Horder[19] found that one of their strains belonged to the buccalis group and one to the fecalis group. Beattie and Yates[29] reported three strains as belonging

[23] Lancet, 1910, 1, p. 1524.
[24] Brit. M. J., 1903, 1, p. 237.
[25] Brit. M. J., 1903, 2, p. 659.
[26] J. M. Res., 1914, 30, p. 487.
[27] J. Infect. Dis., 1914, 14, p. 61.
[28] Arch. Int. Med., 1917, 19, p. 381.
[29] J. Path. & Bacteriol., 1911-1912, 16, p. 247.

to the fecalis group. Lyall[26] reported the fermentation reactions of five organisms isolated from endocarditis. Three of the five fermented mannite and were grouped as fecalis. Quigley[30] examined 6 strains, 4 of which fermented mannite. The organisms reported in the literature are usually streptococci, nonhemolytic in character.

Most of my strains when first isolated were arranged as diplococci. They have a tendency to be lancet shaped but differ from the pneumococcus in not being bile soluble, in not fermenting inulin (with one exception) and in not being capsule producers. Although these strains at times grew out in short chains there is a marked tendency for them to appear as diplococci. In only one strain (5) which displayed hemolytic properties at first, was there any tendency towards spontaneous agglutination in broth cultures.

TABLE 2

Cultural Characteristics of Strains of Streptococcus Viridans

Bile insoluble organisms did not liquefy gelatin, and were not fatal to mice in 2 cc. amounts.

	Plain Broth	Blood Agar	Litmus Milk
1	Turbid diplococci	Green	Acid
2	Turbid short chains	Green	Acid coagulated
3	Turbid short chains	Green	Acid coagulated
4	Turbid short chains	Green	Acid coagulated
5	Clumped at bottom	Hemolytic; indifferent	Acid
6	Turbid diplococci	Green	Acid coagulated
7	Turbid diplococci	Green	Acid coagulated
8	Turbid short chains	Green	Acid coagulated
9	Turbid short chains	Green; indifferent	Acid coagulated
10	Turbid diplococci	Green	Acid
11	Turbid short chains	Green	Acid coagulated
12	Turbid short chains	Green	Acid coagulated
13	Turbid diplococci	Green	Acid coagulated
14	Turbid diplococci	Green; indifferent	Acid

With one exception all my strains produced a green discoloration on the blood agar plate at 24 hours incubation when first isolated. They correspond to the alpha type of Brown.[31] One strain (5) was a typical beta hemolyzer. This strain was found in pure culture in the vegetation on the mitral valve of a patient suffering from subacute endocarditis. Jackson[32] reported the production of typical endocarditis lesions in the myocardium of rabbits by the injection of hemolytic streptococci.

None of the strains liquefied gelatin and all produced acid in litmus milk. The fermentation reactions are recorded in table 3. The mediums used were infusion broth and Hiss serum water with 1% of the carbohydrates listed. Brom-thymol-blue was added to serve as indicator. Readings were taken after two, six and ten days. According to

[30] J. Infect. Dis., 1918, 22, p. 198.
[31] Monograph No. 9, Rockefeller Inst. for M. Research, 1919.
[32] J. Infect. Dis., 1912, 11, p. 243.

Holman's [33] classification three strains were fecalis, four were mitis, one was salivarius, three were nonhemolyticus 1, two were nonhemolyticus 11, one was nonhemolyticus 111. When considering all the sugars used by Brown, however, no classification into groups appears possible on the basis of carbohydrate fermentation.

CULTIVATION OF STRAINS IN PERITONEAL CAVITY OF RABBIT

The strains which figured in the experiments herein described were collected over a period of eighteen months. Consequently it was thought advisable to determine whether prolonged cultivation upon blood agar had any effect on the cultural characteristics and serological reactions of the strains. Two strains were picked at random and placed in collodion sacs in the peritoneal cavity of rabbits. At the end of six

TABLE 3

FERMENTATION REACTIONS

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Glucose	4	2	4	4	4	4	4	4	4	4	4	4	4	4
Lactose	4	2	4	4	4	4	4	4	0	0	4	4	4	0
Sucrose	4	4	4	4	4	4	4	4	4	2	4	4	4	2
Mannite	4	2	2	0	0	0	4	4	4	4	4	0	0	4
Salicin	4	0	0	4	2	4	4	0	4	0	4	0	4	4
Inulin	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Raffinose	4	0	0	4	0	4	0	0	0	4	4	2	4	0

Maximum fermentation 4; small amount fermentation 2; negative 0. All controls were negative.

months they were removed and their characteristics compared with those of the same strains when first isolated and when preserved on blood agar.

The collodion sacs for this purpose were made by heating a piece of glass tubing three centimeters in length and joining it to one end of a one-half ounce horse capsule. After cooling, the capsule was dipped in collodion until a sufficiently durable sac was obtained (five dippings). Then the covered capsule was placed in water bath (50-60 C.) and the liquefied gelatin removed from the sac by means of a capillary pipette. The open end of the glass tube was then plugged with cotton and the sac half filled with water and sterilized in the autoclave. The water was removed and replaced with a 24 hour broth culture of 4 and another with 6. The glass tubes were then sealed off in the flame and the sacs inserted into the peritoneal cavity of a rabbit.

[33] J. Med. Res., 1916, 34, p. 377.

The collodion sac permits the products of bacterial metabolism to diffuse outward and the body fluids to diffuse inward through its walls. At the same time leucocytes cannot pass through the sac to attack the bacteria, thus affording favorable conditions for growth.

After six months in the peritoneal cavity the strains were recovered and found to resemble the homologous strains preserved on blood agar, in cultural characteristics as well as yielding the same serological reactions. It is therefore apparent that whether grown on artificial media or in an animal these characteristics of a particular strain remain unchanged.

SEROLOGIC REACTIONS

Preparation of Immune Serums.—Eleven of the 14 strains isolated from patients suffering from endocarditis were used to immunize rabbits. For this purpose the bacteria were grown in infusion broth (P_H 7.6), and the sediment after centrifugation resuspended in 0.85% NaCl. These suspensions were standardized to a turbidity of one billion per cc., heated to 53 C. for one hour and then preserved in the ice-box.

The rabbits were injected first subcutaneously, then intravenously. On consecutive days 0.25, 0.5, and 1.0 cc. of each suspension was injected subcutaneously into a series of animals. After a rest period of four days intravenous injections were begun and amounts of 0.25, 0.5, and 0.5 cc. were injected. Following another rest period of four days 0.5, 0.75 and 1.0 cc. were injected intravenously, et cetera.

The blood serum of each rabbit was tested from time to time for agglutinin titer and at the end of five weeks the titer was deemed sufficiently high to yield satisfactory results. The serums were obtained by bleeding the rabbits from the heart and preserved by adding an equal volume of 0.6% phenol.

Agglutination.—In determining the titer of each serum the same antigens were used that had served for immunization. To a series of tubes containing dilutions of immune serum ranging from 1 : 20 to 1 : 10,240 was added 0.5 cc. of homologous antigen. The dilutions of serum were made in 0.85% NaCl. The tests were made in the water bath at 50 C. with the depth of the water so adjusted that the level of the liquid in the agglutination tubes stood out somewhat above the surface of the water in the bath, thus bringing into play the slight convection currents within the tubes which appear so effective in the

development of sharp end results in the agglutination reaction. Readings were made after six hours and confirmed after 18 hours in the bath.

Tests were conducted with antigens of living bacteria with practically no difference in results. Controls of normal rabbit serum were used with each antigen. No proagglutinoid zone was observed in any case. The titer of each serum is shown in table 4.

Cross agglutination was tried in the attempt at grouping of the strains. The 11 agglutinating serums which had been prepared were used with 14 strains. Results are found in table 5. The test was performed at dilutions 1 : 10, 1 : 160, 1 : 320, 1 : 500 with uniform results. Each strain was agglutinated only by its homologous serum indicating a high degree of specificity among organisms isolated from patients suffering from endocarditis and possessing similar morphologic and cultural characteristics. This finding is in agreement with Krumwiede and Valentine,[34] Kinsella,[35] Norton,[36] Clawson,[22] Beattie and Yates,[37] Swift and Kinsella,[28] and others. On the basis of agglutination it is quite evident that the strains of Streptococcus viridans discussed in this paper form a heterologous group.

TABLE 4

RESULTS OF AGGLUTINATION WITH HOMOLOGOUS SERUMS

Dilutions of Serum	1	2	3	4	5	6	7	8	9	11	12
1:160	4	4	4	4	4	4	4	4	4	4	4
1:320	3	4	4	4	4	4	4	4	4	4	4
1:640	2	4	4	4	3	3	4	4	4	4	4
1:1280	0	4	2	4	0	2	2	4	4	4	2
1:2560	0	3	2	0	0	0	0	3	3	4	2
1:5120	0	0	0	0	0	0	0	0	2	0	0
1:10240	0	0	0	0	0	0	0	0	0	0	0

Complete agglutination 4; partial agglutination 3, 2; no agglutination 0. All controls were negative.

Absorption of Agglutinins.—Although no cross-agglutination was obtained with the strains investigated, it was deemed advisable to determine whether any absorption of agglutinins might take place. Norton [36] in his work on the relationship of the members of the viridans group found the absorption method more reliable than simple agglutination.

Serums 1, 2, and 4 were tested with negative results. Each of the above mentioned serums was diluted 1 : 50 with a 24 hour broth culture of each of the strains 1 to 14. As a control, the serum was diluted

[34] J. Infect. Dis., *916, 19, p. 217.
[35] Arch. Int. Med., 1917, 19, p. 367.
[36] J. Infect. Dis., 1923, 32, p. 37.
[37] J. Path. & Bacteriol., 1912-1913, 17, p. 538.

1 : 50 with uninoculated broth. The absorption reaction period was four hours in the incubator at 37.5 C. with repeated shaking, and 18 hours in the ice-box. The suspensions were then centrifuged and the sedimented organisms discarded. Each serum which had been exposed to the other strains for absorption was then tested for effects on its titer for its own antigen. No absorption of the agglutinins of the three serums tested had taken place so it was not deemed necessary to continue with the remaining serums.

Precipitin Reactions.—The method of preparation of antigens for the precipitin reaction was a modification of that of Vaughan.[38] Flat bottles containing blood infusion agar were inoculated with 1 cc. of a 24 hour broth culture of strains 1 to 7. After being incubated 48

TABLE 5

Results of Cross Agglutinations

Serums	Antigens													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	4	0	0	0	0	0	0	0	0	0	0	0	0	0
2	0	4	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	4	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	4	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	4	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	4	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	4	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	4	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	4	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	4	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	4	0	0

Complete agglutination 4; no agglutination 0. All controls were negative.

hours at 37.5 C. the growth on the surface of the agar was washed off with 0.85% NaCl and centrifugalized. The organisms were then resuspended successively in 50% alcohol, 70% alcohol, 95% alcohol. The organisms were then placed in a Soxhlet extractor and the fat abstracted with absolute alcohol (one day), then with ether (three days). The residue was ground in an agate mortar and the powder kept in the ice-box. As needed the cellular substance was disrupted in 0.5% KOH (15 times the weight of the powder). The alkali was neutralized with dilute HCl. Precipitation did not occur in as high dilutions as did agglutination with the homologous serums and no cross-precipitation whatever took place.

Complement Fixation Reactions.—The antigens used in these experiments were the same as those used in the immunization of rabbits and

[38] Protein Split Products, 1913.

in the agglutination reactions. All antigens were first tested for hemolytic activity by mixing varying quantities of each antigen with sheep cells and sufficient NaCl solution to bring contents of liquid in all tubes up to a uniform volume. There was no hemolysis in any case. The immune serums were tested for any natural amboceptor against sheep cells by performing the usual fixation test with serum, complement, sheep cells and NaCl solution, but omitting the addition of amboceptor. There was no hemolytic amboceptor present in any of the immune serums.

After going through the preliminaries of obtaining antigenic and anti-complementary doses, the tests were made by incubating at 37 C. It was found that all immune serums gave fixation with homologous

TABLE 6

RESULTS OF COMPLEMENT FIXATION TESTS

	Antigens												
Serums	1	2	3	4	5	6	7	8	9	10	11	12	13 & 14
1.....	2	0	0	0	0	0	0	0	0	0	0	0	0
2.....	0	4	0	0	0	0	0	0	0	0	0	0	0
3.....	0	0	4	0	0	0	0	0	0	0	0	0	0
4.....	0	0	0	4	0	0	0	0	0	0	0	0	0
5.....	0	0	0	0	4	0	0	0	0	0	0	0	0
6.....	0	0	0	0	0	4	0	0	0	0	0	0	0
7.....	0	0	0	0	0	0	2	0	0	0	0	0	0
8.....	0	0	0	0	0	0	0	4	0	0	0	0	0
9.....	0	0	0	0	0	0	0	0	4	0	0	0	0
11.....	0	0	0	0	0	0	0	0	0	0	4	0	0
12.....	0	0	0	0	0	0	0	0	0	0	0	4	0

No hemolysis 4; partial hemolysis 2; complete hemolysis 0. All controls were negative.

antigens. No cross-fixation, however, took place, which result supports those of agglutination and precipitation (table 6). From the serologic tests, it is quite evident that the members of the viridans group worked with form a heterologous group.

Immune Bodies in Patients' Serum.—It is surprising how little work has been done to determine the incidence of immune bodies in the serums of endocarditis patients. Tunnicliff [39] found that opsonins for streptococci were increased in the blood of patients having endocarditis. In the serum of seven of twelve patients, she found agglutinins for the homologous organisms. Kinsella [35] found in testing the blood of 12 patients having subacute bacterial endocarditis that all showed the presence of agglutinins. He used no dilutions over 1 : 40. Clawson [22] tested five cases. In four, agglutinins were found in a dilution of 1 : 50 or more. In one, they could not be detected.

[39] J. Infect. Dis., 1909, 6, p. 346.

I have tested four cases. In three, agglutinins were found in all dilutions up to 1 : 2560 and in the other up to 1 : 5120. In two cases streptococci of the viridans group were isolated from the blood during life, and from the heart blood and vegetations on the mitral valve at necropsy. For all three strains in each case there were agglutinins found in high dilutions of the patients' serum. These cases were all found to be typical at necropsy.

ANIMAL INOCULATIONS

In considering whether the streptococcus may be the etiologic agent in producing bacterial endocarditis, much importance rests on whether lesions similar to human endocarditis lesions can be produced experimentally.

Many investigators have produced endocarditis in rabbits and a few in dogs and monkeys. Poynton and Payne believe that the same organism may produce either simple (rheumatic) endocarditis or malignant (subacute bacterial) endocarditis. Beattie and Dickson [40] report experimental data to show that an organism isolated in a case of subacute bacterial endocarditis may when injected into one animal produce a simple endocarditis and when injected into another animal, a malignant endocarditis. The work of Cole,[41] Davis,[42] Jackson,[32] and Henrici [43] shows that streptococci from other sources are capable of producing endocarditis.

The microscopic myocardial lesions appear to be the most difficult to reproduce. Thalhimer and Rothschild [44] have produced nodular lesions in the myocardium of rabbits by injecting streptococci, but they do not consider these lesions similar to the typical Aschoff nodule. They think their lesions and the lesions of Bracht and Wachter [45] have neither the structure, location, typical cells nor staining reaction characteristic of the Aschoff nodule. But Coombs, Miller and Kettle,[46] Jackson,[32] and Rosenow [27] report that they have produced the Aschoff nodules in the myocardium of rabbits, and that these nodules are typical in all respects of those found in man in acute bacterial endocarditis.

Clawson [22] and Swift [47] give a good description of the pathological picture in man. The gross rheumatic valvular lesion in man consists of

[40] Special Pathology, 1926, 2, p. 507.
[41] J. Infect. Dis., 1904, 1, p. 714.
[42] Ibid., 1912, 10, p. 148.
[43] J. Exper. Med., 1916, 19, p. 572.
[44] Ibid., 1914, 19, p. 429.
[45] Deutsch. Arch. f. klin. Med., 1909, 96, p. 493.
[46] Lancet, 1912, 2, p. 1209.
[47] Swift: J. Exper. Med., 1924, 39, p. 497.

small smooth globular masses on the free margins and at the points of contact of the valves. The microscopic lesions consists of proliferative and exudative inflammation which begins within the valve proper, often some distance beneath the endothelium. The proliferation may be moderate or pronounced. The exudation consists of fibrin and cells, mostly large mononuclear and multinuclear cells though small lymphocytes and a few polymorphonuclear cells are generally present. There is usually a necrotic center in the nodule of inflammation. This necrosis may be found in a degree which can scarcely be noticed or in so extensive a degree that the center of the nodular mass on a valve may have undergone complete necrosis. The endothelium usually ruptures, and a small thrombotic mass from the blood content may collect. The inflammation in a cardiac valve is essentially that of the Aschoff nodule or of a subcutaneous nodule. In the valve, the nodules of inflammation are multiple. With these conditions in mind, the lesions of rabbits were studied to determine whether a similarity existed.

Five of the 14 strains reported in this series were tested in rabbits. The centrifugalized sediment of a 24 hour growth in 100 cc. broth was injected twice a week. In four rabbits injections were intravenous and in the fifth the streptococci were injected directly into the heart, effort being made to deliberately injure the valves to enable the organisms to obtain an early foothold there.

The work with these five strains isolated in cases of bacterial endocarditis agrees with what many investigators have shown, namely, that lesions grossly similar to human endocarditis can be produced in rabbits by injecting streptococci.

Two of the four rabbits injected intravenously over a period of four months showed vegetations on the mitral valves at necropsy. Two rabbits failed to show any evidence of pathological change in the heart valves or myocardium. The removal of the organisms before damage to the heart valves was accomplished, may probably be explained by the high antibody content of the serum of these rabbits. The rabbit which received three large injections directly into the heart at monthly intervals showed vegetations on the mitral and tricuspid valves.

The microscopic appearance of the valvular lesions was similar in all cases and also similar to the lesions studied in several human cases. The lesion seemed to begin within the valve with proliferative and exudative inflammation. Large irregular mononuclear cells with vesicular nuclei were common in the cellular exudate. Multinucleated cells of the same type were frequently found. Edema of the tissues in

the early lesions was pronounced. Necrosis usually occurred in the nodular lesion and extended outward and destroyed the endothelium. Thrombosis in a slight degree occurred in practically all cases. The lesions were essentially the same as the human endocarditis lesions.

TOXIN PRODUCTION

The search for a soluble toxin was pursued in the hope of finding a common poison among the members of a group of organisms isolated from similar pathological lesions, having similar morphological and cultural characteristics but differing in their serological reactions.

Each strain was grown four days in infusion broth at 37.5 C. The cultures were then filtered through a Mandler filter 4 and the filtrates after being tested for sterility were injected into mice. Two cc. of every filtrate failed to kill the animals.

Two strains (4 and 9) were then grown in infusion broth for 14 days and mice injected daily during this period to determine whether toxin might be formed in a longer time than that used in the preceding experiment. No fatalities resulted, however.

Skin tests were also performed on patients suffering from endocarditis. The filtrates of stock strains were injected intradermally in dilutions 1 : 1000, 1 : 500 and undiluted, with negative results. In seven cases the organism isolated from the particular individual tested was used in preparing the filtrates and the tests performed with the same dilutions as above. These also yielded negative results. Readings were taken 6 and 24 hours after injection. It appears from these results that no real exotoxin is secreted by these members of the viridans group associated with endocarditis.

It was thought that possibly an endotoxin or split protein product derived from the bacterial cell might be the factor giving rise to the pathological condition in endocarditis. Hence the attempt was made to liberate this apparent poisonous substance from the viridans cell by means of proteolytic enzymes. A 1% solution of trypsin in 0.85% NaCl was passed through a Mandler filter, 4. This filtrate was mixed with an equal volume of a heavy suspension of strain 11 in buffer solution (P_H 7.6), containing a 24 hour growth of 11 in 100 cc. broth which was centrifugated and resuspended in 5 cc. of the buffer solution. Mice were injected with 0.5 cc. and 1 cc. of this mixture daily for a period of ten days. All the mice lived. Guinea-pig serum and the filtrates of broth cultures of proteolytic organisms as Serratia prodigiosa (Bacillus prodigiosus), Proteus vulgaris and other gram

negative bacilli capable of liquefying gelatin were used in place of trypsin. Only negative results were obtained. The same experiments were tried using 4 and 8, but all efforts to liberate an endotoxin or poisonous split protein were unavailing.

SUMMARY

In a relatively high percentage of cases of endocarditis a streptococcus can be isolated from the blood; and also frequently from vegetations on heart valves and infarcts in cases coming to necropsy.

In the cases studied, all but one yielded an organism producing green colonies on blood agar. In the one exception a typical beta hemolyzer was isolated.

None of the strains liquefied gelatin.

All strains produced acid in litmus milk.

The fermentation of carbohydrates was found to be irregular. No satisfactory classification can be made on this basis.

The streptococci are insoluble in bile and of low virulence.

Cultivation of organisms in collodion sacs in the peritoneal cavity of rabbits did not change their morphologic and cultural characteristics, or their serologic reactions.

There occurred no cross-agglutination, cross-precipitation or cross-complement fixation among the strains isolated. From these observations and the fact that no absorption of agglutinins took place, it appears impossible to group these streptococci on the basis of their serological reactions.

Immune bodies for the homologous strain were found in the blood of all patients tested.

Lesions of the heart valves similar to those occurring in human beings can be produced experimentally in animals by injection of these organisms directly into the heart and intravenously.

It appears that no exotoxin, endotoxin, or poisonous split protein can be liberated from the strains studied.

When the foregoing facts are considered, there seems to be ground for the belief that endocarditis with its associated lesions is produced by streptococci, usually members of the viridans group. This statement is based on the fact that inoculation of rabbits with cultures of the groups studied has led to the development of valvular lesions comparable to those seen in human beings.

PRECIPITATION WITH FRACTIONS OF SYPHILITIC SERUM AND ARACHNOID FLUID

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An understanding of the phenomenon of precipitation between syphilitic serum or arachnoid fluid and tissue extract antigen is dependent upon a knowledge of the specific substances between which the reaction takes place. The first step toward this knowledge is the determination of the particular fraction of the syphilitic fluid containing, or associated with, the substances which react with antigen. This has been attempted by several workers. The results, however, have shown considerable disagreement.

Sahlmann [1] fractionated syphilitic serum with carbon dioxide, also with weak hydrochloric acid, and reported stronger results with the albumin than with the globulin fraction in the Sachs-Georgi test. Mackie,[2] following up this work, concluded that the pseudoglobulin fraction which remained in solution in the presence of carbon dioxide contained practically all the substances which gave flocculation. Herrold [3] employed ammonium sulfate for fractionating both serum and spinal fluid and tested the fractions with his ring modification of the precipitation test to determine the presence of antibody. He reported that the euglobulin fraction contained almost all the syphilitic antibody. More recently, Schuiringa and Kapsenberg [4] studied the rôle of the globulins and albumins in the Sachs-Georgi reaction. They found that the globulin from a syphilitic serum precipitated by half saturation with ammonium sulfate gave a positive reaction while the albumin gave a negative reaction. The varying results obtained by these different workers indicated the need for further study of the problem.

This paper reports a study of the relative amount of precipitin reacting substances in the globulin and albumin fractions of syphilitic serum and arachnoid fluid.

PROCEDURE

It is generally accepted that 33⅓% saturation of serum with ammonium sulfate throws down mainly the euglobulin fraction, 50% saturation the total globulin and 100%, both the globulin and albumin fractions. These proportions

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[1] Ztschr. f. Immunitätsforsch., 1922, 33, p. 130.
[2] J. Path. & Bact., 1923, 26, p. 120.
[3] J. Am. M. A., 1923, 81, p. 203.
[4] Ztschr. f. Immunitätsforsch, 1925, 42, p. 300; Abstr. Bact., 1925, 9, p. 435.

of ammonium sulfate with serum, followed by thorough centrifugalization, were used in the separation of the several protein fractions of the serum.

For determining the precipitin reacting substances of the protein fractions, the Kahn precipitation test[5] was employed (the "quantitative" procedure for the serum fractions and the "spinal fluid" procedure for the various dilutions of the arachnoid fluid fractions). The Kahn test seemed particularly well adapted to the study because of its relative simplicity and rapidity of results.

When saturating serum in varying degrees with ammonium sulfate, particularly beyond 50%, difficulty was experienced in separating the precipitate from the medium by centrifugation. This was overcome in two ways. First, strongly potent serums were selected and diluted with salt solution before use in the experiments. Second, talc was used to assist the separation of the protein during centrifugation. Talc was employed also in the arachnoid fluid experiments. It was found that talc in no way interfered with the final reaction.

After saturating the diluted serum or spinal fluid in varying degrees with ammonium sulfate, the mixtures were placed in the water bath at 56 C. for 15 minutes to hasten precipitation of the protein. Experiments showed that the sulfate-serum mixtures could be heated even to 2 hours without affecting the final reaction.

The use of ammonium sulfate in connection with the experiments made it necessary to standardize the Kahn antigen in the presence of this salt as is done in the case of the Kahn spinal fluid procedure.

EXPERIMENTS WITH SERUM FRACTIONS

Euglobulin and Pseudoglobulin Fractions.—Three 2 cc. amounts of strongly positive syphilitic serum previously diluted 1 : 10 with physiologic salt solution were pipetted into three centrifuge tubes and saturated solution of ammonium sulfate added to make 33⅓, 40 and 50% saturations, respectively. After thorough mixing, these three tubes and the serum control were placed for 15 minutes in a 56 C. water bath. After thorough centrifugalization, the supernatant fluid of the euglobulin precipitate obtained with 33⅓% saturation, was removed and brought up to 50% saturation in order to obtain the pseudoglobulin fraction. Each of the globulin precipitates was dissolved in 2 cc. physiologic salt solution, an amount equal to the volume of serum to which the ammonium sulfate was added. The globulin solutions and the original serum were tested by the Kahn quantitative procedure.

It was found that the euglobulin fraction obtained after 33⅓% ammonium sulfate saturation gave considerably weaker reactions than the fraction obtained after 40% saturation; this fraction, in turn, gave slightly weaker reactions than the 50% fraction and this latter fraction gave results similar to the original serum. The euglobulin fraction and the pseudoglobulin fractions gave reactions of comparable strength. Table 1 gives the results of this experiment with two serums.

[5] Kahn, R. L.: Serum Diagnosis of Syphilis by Precipitation: Governing Principles, Procedure and Clinical Application of the Kahn Precipitation Test, 1925.

It is evident from this table that the combination of euglobulins and pseudoglobulins contained the largest number of syphilitic reacting substances and that the reacting substances were present in both the euglobulin and pseudoglobulin fractions.

Globulin and Albumin Fractions.—It was desired next to determine whether the syphilitic reacting substances were contained only in the globulin fractions or were present also in the albumin fraction. In order to determine this, a study of the following four fractions of syphilitic serum in comparison with the original serum was attempted: globulin, resulting from 50% saturation with ammonium sulfate; globulin plus some albumin, resulting from 75% saturation with ammonium sulfate; globulin plus albumin, resulting from 100% saturation with ammonium sulfate; albumin, resulting from complete saturation with ammonium sulfate of the supernatant fluid of the globulin precipitate.

TABLE 1

PRECIPITATION REACTIONS WITH VARIOUS GLOBULIN FRACTIONS

% Saturation of Diluted Serum with $(NH_4)_2SO_4$	Resulting Serum Fraction	Serum 1 — Serum Dilutions in Kahn Quantitative Test					Serum 2 — Serum Dilutions in Kahn Quantitative Test				
		1:10	1:30	1:50	1:70	1:90	1:10	1:30	1:50	1:70	1:90
33⅓	Euglobulin	++++	±	0	0	0	++++	++++	+	0	0
40	Eu. + part of pseudoglobulin	++++	++++	±	0	0	++++	++++	++++	+	0
50	Total globulin	++++	++++	±	0	0	++++	++++	++++	+++	++
50 (supernatant of euglobulin precipitate)	Pseudoglobulin	++++	±	0	0	0	++++	±	0	0	0
0 (control)	Whole serum	++++	++++	±	0	0	++++	++++	++++	++	0

Highly potent syphilitic serum was diluted 1:50 with salt solution. Two cc. of this diluted serum were measured into each of three centrifuge tubes. Two cc. saturated ammonium sulfate solution were added to tube 1 (50% saturation); 6 cc. saturated solution to tube 2 (75% saturation) and crystals of ammonium sulfate to tube 3 (until completely saturated). The three tubes were placed for 15 minutes in a 56 C. water bath to hasten precipitation. Small amounts of talc were added to each tube, the contents thoroughly mixed, and centrifugalized for half an hour.

The supernatant fluid from the 50% saturated solution (1) was pipetted into another tube (4), completely saturated with ammonium sulfate crystals and heated at 56 C. for 15 min. to precipitate the albumin fraction. A small amount of talc was added and the mixture centrifugalized. The supernatant fluids of tubes 2, 3 and 4 were then pipetted off and discarded. To the residues of tubes 1, 2, 3 and 4, respectively, were added 2 cc. amounts of salt solution to dissolve the protein precipitates. The mixtures were again centrifugalized to throw down the talc. In tube 1 was the total globulin fraction; in tube 2 the globulin fraction and some of the albumin fraction; in tube 3 the globulin and albumin fractions, and in tube 4, the albumin fraction alone.

The four fractions and the diluted whole serum were then tested comparatively for precipitin substances. The results with one of the serums tested are recorded in table 2.

TABLE 2

PRECIPITATION REACTIONS WITH GLOBULIN AND ALBUMIN FRACTIONS AND UNFRACTIONATED SERUM

% Saturation of Diluted Serum with $(NH_4)_2SO_4$	Resulting Serum Fraction	Dilutions Used in Kahn Quantitative Test					
		1:50	1:75	1:100	1:125	1:150	1:200
50	Globulin	++++	+++	++	+	0	0
75	Globulin + part of albumin	++++	+++	+	±	0	0
100	Total globulin + albumin	++++	+++	+	±	0	0
100 (supernatant fluid of globulin precipitate)	Albumin	0	0	0	0	0	0
0 (control)	Whole serum	++++	+++	+	±	0	0

The findings indicated close agreement in the number of reacting substances in the globulin fraction, the combined globulin albumin fraction and the original serum. The fact that no increase in strength of reaction was observed due to the presence of the albumin fraction, suggested that all the substances were contained in the globulin fraction. Fraction 4, containing the albumins alone gave negative results, which corroborated the findings with fractions 1, 2 and 3.

Since the serums employed in this experiment had been diluted 1 : 50 with saline, it seemed possible that if the albumin fraction were concentrated to correspond with a lower dilution of the serum, some reacting substances might be demonstrated. The following experiment was therefore carried out.

Concentrated Albumin Fraction.—A serum giving a precipitation reaction in a dilution of 1 : 100 was selected. Five cc. of this serum diluted 1 : 10 with salt solution were measured into a centrifuge tube and thoroughly mixed with 5 cc. of saturated ammonium sulfate. The mixture was heated for 15 minutes in a water bath at 56 C., shaken thoroughly with a little talc and centrifugalized for 30 minutes. Ten cc. of the clear supernatant fluid (corresponding to 0.5 cc. undiluted serum) was saturated completely with ammonium sulfate crystals. The mixture was heated for 15 minutes at 56 C. and after the addition of talc, thoroughly centrifugalized. The supernatant fluid was pipetted off and 1 cc. salt solution added to the talc albumin precipitate, mixed thoroughly to dissolve the albumin and again centrifugalized to throw down the talc. This supernatant fluid thus contained the total albumin fraction of 0.5 cc. of serum in 1 cc. of saline. The results with one syphilitic and two nonsyphilitic serums are given in table 3.

TABLE 3

PRECIPITATION REACTIONS WITH ALBUMIN FRACTION OF SERUMS

Serum Number	Fraction Tested	Dilutions Used in Kahn Quantitative Test					
		1:2	1:5	1:10	1:20	1:50	1:100
1	Albumin	±	0	0	0	0	0
	Serum control	++++	++++	++++	++++	++++	0
2	Albumin	+	±	0	0	0	0
	Serum control	0	0	0	0	0	0
3	Albumin	+	±	0	0	0	0
	Serum control	0	0	0	0	0	0

It is evident from this table that no reactions of significance were given by the concentrated albumin fractions although concentrated ten times the dilution in which the serum reacted. The weak reactions with the albumin fraction in both the positive and negative serums are undoubtedly due to the presence of relatively large amounts of ammonium sulfate associated with the concentration of the albumin fraction.

EXPERIMENTS WITH FRACTIONS OF ARACHNOID FLUID

Method.—Into each of four 15 cc. centrifuge tubes were measured 2 cc. of a pooled syphilitic spinal fluid. Sufficient saturated solution of ammonium sulfate was added to the first three amounts to make a final saturation of 33⅓, 50, and 75%, respectively. The fourth amount was completely saturated with ammonium sulfate crystals. After thorough mixing of the contents, these four tubes were placed for 15 minutes in a water bath at 56 C. After the addition of a small amount of talc to each, the four tubes were centrifugalized. The precipitate resulting from 33⅓% saturation was considered the euglobulin

fraction; from 50% saturation, the total globulin; from 75%, the total globulin plus a part of the albumin; from 100% saturation, the total globulin plus albumin.

It was desired also to separate out the pseudoglobulin and the albumin fractions. To obtain the pseudoglobulin fraction, the supernatant fluid of the euglobulin precipitate was removed and brought up to 50% saturation. To obtain the albumin fraction, the supernatant fluid of the total globulin precipitate was removed and completely saturated with ammonium sulfate crystals. These two tubes were treated in the same manner as the previous four. The supernatant fluid was removed from all tubes with a capillary pipet, leaving the least possible amount of ammonium sulfate. Each of the 6 precipitates was dissolved in an amount of salt solution equal to 1/10 the original volume of fluid. All tubes were centrifugalized to throw down the talc and the resulting clear solutions were used in the tests.

TABLE 4

PRECIPITATION REACTIONS WITH GLOBULIN AND ALBUMIN FRACTIONS OF ARACHNOID FLUID

% Saturation of Arachnoid Fluid, with $(NH_4)_2SO_4$	Resulting Fraction (In 1/10 Original Volume of Fluid)	Spinal Fluid 1 — Dilutions in Kahn Quantitative Test					Spinal Fluid 2 — Dilutions in Kahn Quantitative Test				
		1:1	1:2	1:4	1:8	1:16	1:1	1:2	1:4	1:8	1:16
33⅓	Euglobulin	++++	++++	++	±	0	+++	+	0	0	0
50 (supernatant fluid from euglobulin precipitate)	Pseudoglobulin	++++	++++	++	±	0	++++	++++	+	0	0
50	Total globulin	++++	++++	++++	++	±	++++	++++	+++	±	0
75	Globulin + part of albumin	++++	++++	++++	+	±	++++	++++	+++	0	0
100	Globulin + albumin	++++	++++	++++	+	±	++++	++++	+++	0	0
100 (supernatant fluid from euglobulin precipitate)	Albumin	0	0	0	0	0	0	0	0	0	0

Fractions of Arachnoid Fluid.—The solutions of euglobulin, pseudoglobulin, total globulin, and albumin were tested in dilutions 1 : 1, 1 : 2, 1 : 4, 1 : 8, and 1 : 16. In each case 0.15 cc. of each dilution was tested with 0.01 cc. standard antigen dilution. In table 4 are recorded the findings with 2 fluids which are typical of the series studied.

These results corroborate the findings with serum. There is close agreement in the amount of reacting substances in the total globulin and the combined globulin-albumin fraction. In most fluids studied, both the euglobulin and pseudoglobulin fractions of the spinal fluids gave reactions. No reaction was obtained with the albumin.

SUMMARY

An attempt was made to determine which fraction of syphilitic serum and arachnoid fluid contains the reacting substances responsible for precipitation with beef heart antigen. The fractions studied were obtained by various degrees of saturation with ammonium sulfate. The precipitin reacting substances were determined quantitatively by means of the Kahn test.

The data indicates that the reacting substances are associated with the globulin fraction and not at all with the albumin, and that both the euglobulin and pseudoglobulin fractions of the globulin contain these reacting substances.

A CULTURE MEDIUM FOR DIFFERENTIATING ORGANISMS OF TYPHOID-COLON AEROGENES GROUPS AND FOR ISOLATION OF CERTAIN FUNGI *

WITH COLORED PLATE

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It has been shown, by Koser,[1] that bacteria of the colon-aerogenes groups may be separated according to their ability to utilize citrate; and that this phenomenon is correlated more closely with the source of the cultures than either the methyl red or Voges-Proskauer tests. The test is based on the inability of the intestinal Bacterium coli (Escherichia coli) to utilize citric acid, or sodium citrate, as a source of carbon; and the ability of organisms of the aerogenes section to use the citrate and grow luxuriantly. The mediums recommended by Koser[1] are clear watery solutions of certain inorganic salts, an inorganic source of nitrogen and a citrate as the only source of carbon, as for example the following: NaCl, 5 Gm.; $MgSO_4$, 0.2 Gm.; (NH_4) H_2PO_4, 1 Gm.; K_2HPO_4, 1 Gm.; sodium citrate, 2 Gm. (2.77 Gm. sodium citrate (5½ H_2O) in 1,000 cc. of distilled water. The reaction of such a solution should be about P_H 6.6 or 6.8. In this medium typical strains of fecal Bact. coli do not grow and the inoculated fluid remains clear after inoculation at 30 or 37 C.; however, Bact. aerogenes (Aerobacter aerogenes) and related organisms reproduce rapidly in it, producing a turbidity in 24 to 48 hours.

While the fluid citrate medium has given excellent results in my hands, there has been at times some confusion due to the turbidity produced by the bacteria inoculated, even when the latter had not increased in numbers. This observation led to the belief that a solid medium containing an indicator might obviate this disadvantage, since bacterial growth on such a solid medium is always clearly indicated by colony formation, and usually by a striking change in the color of the indicator.

After the trial of several different combinations the following medium, which is essentially Koser's citrate solution with the addition of agar and brom-thymol blue, seemed to best overcome the difficulty mentioned:

Citrate agar medium	Gm. or CC.
Agar, clean and dry	20
NaCl	5
$MgSO_4$	0.2
$(NH_4)H_2PO_4$	1
K_2HPO_4	1
Sodium citrate (2.77 Gm. sodium citrate 5½ H_2O)	2
Distilled water	1,000
Brom-thymol blue (1.5% alcoholic)	10

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[1] J. Bact., 1924, 9, p. 59.

After dissolving the various salts in sterile distilled water add 20 Gm. of clean, washed agar and sterilize the mixture at 15 pounds pressure for 15 minutes in an autoclave. After adjusting the reaction of the citrate agar to P_H 6.8, add the indicator, di-brom-thymol-sulphon-phthalein (brom-thymol blue). Variations have been observed in the intensity of the color reactions obtained with lots of brom-thymol blue distributed by different commercial firms. Uniformly satisfactory results have been obtained by using the indicator marketed by National Anilin and Chemical Company or Synthetical Laboratories, 10 cc. of a 1.5% alcoholic solution in 1,000 cc. of citrate agar. The transparent, olive-green medium is then distributed in test tubes and allowed to solidify in a slanted position. Since brom-thymol blue is an indicator having a color range from yellow at P_H 6. to blue at P_H 7.6, the color of the citrate agar becomes yellow or orange when sufficient acid is produced, and turns Prussian blue when enough alkali is present.

A 0.4% aqueous solution of brom-thymol blue may be used in the proportion of 20 cc. to 1000 cc. of citrate agar, instead of the alcoholic indicator solution. The aqueous solution is prepared[1a] by grinding one decigram (0.1 gram) of dry powdered brom-thymol blue in an agate mortar with 3.2 cc. of N/20 NaOH and when solution is complete, dilute to 25 cc. with water. The brom-thymol blue distributed by The Lamotte Chemical Products Co. gives uniformly satisfactory results when used in these proportions.

It is realized that the use of agar, because of its organic nature, might be objected to in a test made solely to determine the ability of organisms to utilize citrate; and that some bacteria are able to reproduce on a simple jelly composed only of agar and water. However, trial has shown that this objection has no practical bearing on the use of citrate agar as a differential medium.

DIFFERENTIATION OF COLON-AEROGENES GROUP

Several hundred strains of Bact. coli and of Bact. aerogenes have been used in comparing the differential value of the new citrate agar with that of Koser's liquid citrate medium. Consistently parallel results were obtained with the two mediums after incubation at either 30 or 37 C. All of the strains of Bact. aerogenes, which grew and produced a turbidity in the fluid medium, grew luxuriantly on the surface of citrate agar, forming large, raised, moist, confluent, grayish or greenish blue colonies; and changed the medium from its initial green to a deep Prussian blue color in 24 to 48 hours. Visible growth did not occur in the stab inoculations and no gas was formed.

[1a] Clark: The Determination of Hydrogen Ions; 1925, p. 80.

Fecal strains of Bact. coli, which failed to produce a turbidity of the citrate solution, either failed to grow or, as occurred with some strains, only developed as hardly visible pinpoint colonies on citrate agar without changing the color of the medium. When clumps, or large amounts of Bact. coli, were used for inoculation these masses became dry, shrunken and light yellowish tan in color without effecting the color of the underlying medium. The olive green color of the media inoculated with Bact. coli furnished a striking contrast with the deep blue color produced by Bact. aerogenes.

The citrate agar described is of practical value for the differentiation of bacteria of the colon-aerogenes group. It has consistently given results, comparable to those obtained with the liquid citrate medium, and for routine work it has certain advantages including: the ease with which growth may be determined and the striking blue color produced by Bact. aerogenes.

DIFFERENTIATION OF THE TYPHOID-PARATYPHOID GROUPS

A number of investigators, including Wagner,[2] Altobelli,[3] Ayers, Rupp and Johnson,[4] Pesch,[5] and Koser,[6] have noted the ability of certain organisms of the typhoid-colon-aerogenes groups to utilize organic acids or their salts as sources of carbon. It is known that Salmonella schotmülleri (B. paratyphosus B), Salmonella (or B.) enteritidis, and others can utilize citrate, producing alkaline carbonates and bicarbonates. On the other hand, Eberthella typhi (B. typhosus) and Salmonella paratyphi (B. paratyphosus A) neither grow nor change the reaction of citrate mediums.

The citrate agar medium containing brom-thymol blue, advocated for the differentiation of the colon-aerogenes group, is also useful for separating certain bacteria of the typhoid-paratyphoid groups.

A large number of strains of the latter groups of bacteria have been inoculated on citrate agar, using the fluid citrate medium in each instance as a control. As shown in table 1, Eb. typhi, Eb. dysenteriae, Eb. paradysenteriae (Flexner and Hiss-Russell), Salmonella paratyphi, Salmonella pullora and Salmonella morgani failed to grow in either medium and did not change the color of the citrate agar medium. On the other hand, Salmonella schotmülleri, aertrycke, enteritidis and typhi-murium, grew luxuriantly in 24 to 48 hours, producing a cloud in the fluid

[2] Centralbl. f. Bakteriol., I. O., 1913, 71 p. 25.
[3] Società Toscana d'Igiene, 1914.
[4] U. S. Dept. Agricult. Bull. 782, 1919.
[5] Centralbl. f. Bakteriol., I. O., 1921, 86, p. 97.
[6] J. Bact., 1923, 8, p. 493.

TABLE 1

GROWTH OF VAROUS ORGANISMS OF THE TYPHOID-PARATYPHOID GROUPS ON CITRATE AGAR CONTAINING BROM-THYMOL BLUE, AT 37.5 C. FOR 48 HOURS

Organism	Growth on Surface	Growth on Stab	Gas	Reaction of Medium	Growth in in Koser's Fluid Citrate Medium
Eberthella typhi, 8 strains	0	0	0	Unchanged, olive green	0
Eberthella dysenteriae, Shiga, 1 strain	0	0	0	Unchanged	0
Eberthella paradysenteriae, Flexner, 1 strain	0	0	0	Unchanged	0
Eberthella paradysenteriae, Hiss-Russell, 1 strain	0	0	0	Unchanged	0
Salmonella paratyphi (B. paratyphosus A.), 5 strains	0	0	0	Unchanged	0
Salmonella morgani, 1 strain	0	0	0	Unchanged	0
Salmonella pullora, 1 strain	0	0	0	Unchanged	0
Salmonella schotmülleri (B. paratyphosus B.), 14 strains	++++	0	0	Alkaline, deep Prussian blue	++++
Salmonella aertryeke, 6 strains	++++	0	0	Alkaline, deep Prussian blue	++++
Salmonella enteritidis, 5 strains	++++	0	0	Alkaline, deep Prussian blue	++++ pellicle
Salmonella typhimurium	++++	0	0	Alkaline, deep Prussian blue	++

0, no growth; ++, moderate growth; ++++, luxuriant growth, in all tables.

TABLE 2

GROWTH OF VAROUS UNRELATED ORGANISMS ON CITRATE AGAR CONTAINING BROM-THYMOL BLUE, AT 37.5 C. FOR 48 HOURS

Organism	Growth on Surface	Growth on Stab	Gas	Reaction of Medium	Growth in in Koser's Fluid Citrate Medium
Staphylococcus aureus, 2 strains	0	0	0	Unchanged, olive green	±
Streptococcus pyogenes, 2 strains	0	0	0	Unchanged	±
Diplococcus pneumoniae, 1 strain	0	0	0	Unchanged	0
Neisseria intracellularis, 1 strain	0	0	0	Unchanged	±
Clostridium tetani, 1 strain	0	0	0	Unchanged	0
Clostridium welchii, 1 strain	0	0	0	Unchanged	0
Lacto bacillus acidophilus	0	0	0	Unchanged	0
Lacto bacillus bulgaricus	0	0	0	Unchanged	0
Lacto bacillus odontolyticus	0	0	0	Unchanged	0
Corynebacterium xerosis, 1 strain	0	0	0	Unchanged	0
Corynebacterium, hoffmani, 1 strain	±	0	0	Unchanged	+
Corynebacterium diphtheriae, 1 strain	±	0	0	Unchanged	+
Vibrio comma, 1 strain	0	0	0	Unchanged	0
Proteus vulgaris, 1 strain	++	0	0	Alkaline, Prussian blue slant	++
Uninoculated controls	0	0	0	Unchanged, olive green	0

±, questionable growth.

medium, and forming translucent greenish blue-green colonies on citrate agar, and producing sufficient alkali to color the latter medium a deep Prussian blue. Because of the striking cultural results just described, citrate agar is a valuable aid in the identification of B. paratyphosus B. and related organisms. Since this medium inhibits the growth of Bact. coli and many other unrelated bacteria (table 2), it is quite possible that it might prove of practical value for the isolation of organisms of the Salmonella group, directly from feces, in cases of so-called food poisoning.

TABLE 3

GROWTH AT 37.5 C. VARIOUS FUNGI AND FUNGI IMPERFECTI ON CITRATE AGAR CONTAINING BROM-THYMOL BLUE

Organisms	Growth on Surface	Growth in Stab	Color Reaction of Medium *
Mucor vulgaris, 2 strains—Cox	++++ dry, brown	0	Faint yellow, later faint blue slant
Saccharomyces cerevisiae, 4 strains	++++ yellow or orange	0	Yellow or orange
Saccharomyces ellipsoideus	+ yellow	0	Yellow
Aspergillus niger, 3 strains—Cox	++++ brownish black	0	Yellow—later faint blue
Torulahistolytica, 2 strains	++++ Confluent moist yellow	0	Yellow
Cryptococcus, unclassified, 2 strains	0	0	Unchanged, olive green
Trichophyton crateriforme	0	0	Unchanged, olive green
Trichophyton equinum	0	0	Unchanged, olive green
Monilia psilosis Ashford, 8 strains	Yellow orange		Yellowish orange
Monilia, unclassified Ashford, 8 strains			
Monilia tropicalis Cast. Cox			
Monilia kruseii, Cort., Cox			
Monilia macedoniensi, Cort, Cox			
Monilia albicans, Cox			
Madurella mycetoma, Scott	++ yellow	0	Yellow
Sporotrichuris schenki, 2 strains	++ yellowish orange	0	Yellow
Yeast from root abscess of tooth	++++ yellow	0	Yellow
Yeast from sputum	++++ yellow	0	Yellow

* Medium, olive green at P_H 6.8, becomes blue when more alkaline and yellow or orange when more acid.

THE GROWTH OF CERTAIN FUNGI ON CITRATE AGAR

A number of fungi, and fungi imperfecti, grew luxuriantly and produced striking color changes on citrate agar containing brom-thymol blue. As is shown in table 3, no attempt was made to describe the individual morphological variations of the colonies, attention being paid mainly to the amount of growth which occurred and to the color reactions. All of the organisms tested in this group excepting two strains each of cryptococcus and trichophyton, grew on citrate agar and produced sufficient acid to give a yellow or orange color to the medium.

Twenty strains of Monilia, including eight of Monilia psilosis, obtained from Ashford, all grew luxuriantly forming bright yellow or orange colonies on the surface, and changed the medium from green to a rich yellowish-orange color. This yellow, or acid, color reaction of the Monilia colonies and of the medium furnished a decided contrast to the blue, or alkaline, reaction produced by the intestinal bacteria which grow on citrate agar, thus aiding in the identification and isolation of the former in cultures from feces. The inhibition of the growth of Bact. coli and of a number of other bacteria on this medium is an additional factor favorable to its use for the isolation of various fungi from pathological materials contaminated with bacteria. It is hoped that further work will be carried out along these lines.

SUMMARY

A solid citrate agar medium has been prepared by adding agar and brom-thymol blue to one of the citrate solutions recommended by Koser.

Escherichia coli was markedly or completely inhibited on this citrate agar and did not change the color of the medium; while Aerobacter aerogenes grew luxuriantly forming large raised greenish-blue colonies and produced a blue color in the medium. The results were in every test comparable to those obtained with the fluid citrate medium.

Eberthella typhi, Eberthella dysenteriae and paradysenteriae, Salmonella paratyphi, Salmonella pullora and Salmonella morgani failed to grow or change the color of citrate agar; while Salmonella schotmülleri, Salmonella aertrycke, Salmonella enteritidis and Salmonella typhi-murium grew luxuriantly forming translucent greenish-blue colonies and changing the color of the medium to a deep Prussian blue.

A number of unrelated organisms, including Escherichia coli, and a number of pyogenic cocci, etc., failed to grow on citrate agar.

Certain fungi and fungi imperfecti, including a number of sacchyromyces, 20 strains of Monilia, 2 of Sporotrichum schenki and 1 of Madurella mycetoma, grew luxuriantly on citrate agar forming yellow or yellowish-orange colonies and changing the color of the medium to a deep yellow or orange.

CONCLUSION

The citrate agar described in this paper is a valuable medium for practical use; in differentiating Escherichia coli from Aerobacter aerogenes; in separating Salmonella schotmülleri and related organisms from Salmonella paratyphi, Eberthella typhi, and Eberthella dysenteriae; and as an aid in the isolation and identification of certain fungi and fungi imperfecti.

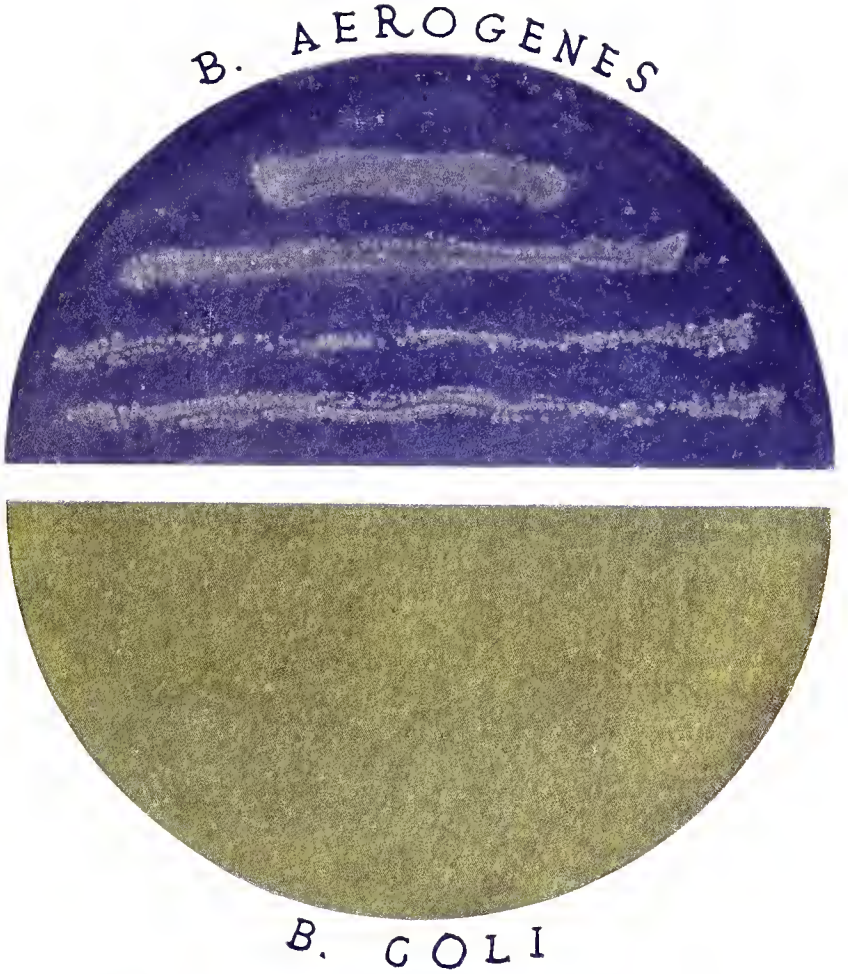

Fig. 1.—Color reaction of citrate agar with brom-thymol blue indicator: B. aerogenes, blue; B. coli, unchanged olive green.

Fig. 2.—Color reaction of citrate agar with brom-thymol blue indicator: B. paratyphosus B blue; B. typhosus and B. paratyphosus A, unchanged olive green.

A COMPARISON OF STRAINS OF BRUCELLA ABORTUS ISOLATED FROM MAN WITH THOSE FROM CATTLE

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Recent literature has contained reports of cases of undulant fever in man from which cultures of an organism indistinguishable from Brucella abortus have been recovered. This has prompted investigators to discover, if possible, the source of this infection. In 1918 Evans [1] reported that Brucella abortus and Brucella melitensis are closely related in all of their biological characteristics and that Brucella abortus is only a variety of Brucella melitensis. She has found, however, that by means of the agglutinin absorption test the two strains may be differentiated with certainty.

During the last year the author has had the opportunity to study five strains of Br. abortus that he has isolated from the blood and urine of man and to compare them with strains of Brucella abortus recovered from bovine fetus, placenta, and milk. They have been examined with reference to their atmospheric requirements, as evidenced by their original isolations, their serologic relationship, their pathogenicity for ginea-pigs, their effect upon pregnant cattle when injected intravenously, and their ability to establish themselves in the udders of cattle.

When Bang [2] first described Brucella abortus as the cause of infectious abortion in cattle, he stated that it had anaerobic tendencies and grew better in an atmosphere with a reduced amount of oxygen. Still further work has shown that it is not a reduction in oxygen that facilitates its growth but rather an increased amount of carbon dioxide. Smith and Fabyan [3] and Schroeder and Cotton [4] observed that the Bang organism was pathogenic for guinea-pigs. It produced a chronic progressive disease similar to tuberculosis, except that the majority of the guinea-pigs did not lose weight or die and some pigs evidently recovered from the infection.

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[1] J. Infect. Dis., 1918, 22, p. 580.
[2] Ztschr. f. Thiermed., 1897, 1, p. 241.
[3] Centralbl. f. Bakteriol., I, O., 1912, 61, p. 549.
[4] 28th Annual Report, U. S. B. A. I., 1911, p. 139.

Although Brucella abortus occurs more frequently in cattle than in any other species of domestic animal, it has been recovered from sheep, horses and swine. Quite extensive outbreaks of abortion in swine due to Brucella abortus have been reported. The porcine strains that have been isolated are reported to grow readily upon nutrient agar without an increased amount of CO_2 or sealing of the tube. They are also considered to be more pathogenic for guinea-pigs than the bovine strains. According to Smith [5] the primary lesions produced in the tissues of guinea-pigs by both strains are identical, but as the disease progresses the porcine strain produces a lesion which shows a more extensive infiltration with polymorphonuclear leucocytes often followed by a central necrosis. Nearly all workers have produced abortion in cattle by injecting the bovine strain intravenously into pregnant cattle, but abortion has not been produced regularly in cattle by injecting porcine strains intravenously.

One of the difficulties encountered in studying the comparative virulence of various strains of Brucella abortus is a decreased pathogenicity of the organism for guinea-pigs after it has been cultivated on culture medium for various periods of time. Huddleson,[6] as well as many other observers, has reported strains that at one time produced typical lesions of Br. abortus infection in guinea-pigs while perhaps a year later the same strain was nonvirulent for guinea-pigs. Porcine, as well as bovine, types have shown this same tendency. Whether this is true of the strains isolated from man cannot be stated at this time. The biological characteristics of only five of these strains have been studied.

The recovery of the organism from man was made by guinea-pig injection and by cultures from the blood and urine on nutrient agar to which sterile horse serum had been added. The cultures were made by adding to the medium from 1 to 3 cc. of blood from the patient. The tubes were sealed with sealing wax and incubated at 37 C. Four of the strains were recovered in this manner while the fifth was grown in an atmosphere where 15% of the air had been replaced by 10% of CO_2. The first generation of these strains has always been difficult to grow when the culture tubes are sealed with wax. In the majority of instances no growth has developed for 6 or 8 days. It has also been necessary to open the tubes once or twice and smear the mixture of blood and condensation water over the slanted surface of agar to obtain

[5] J. Exper. Med., 1926, 43, p. 207.
[6] Technical Bulletin 55, Agric. Exper. Sta., Mich. Agric. College, 1922.

an observable growth. The second and following generations, however, have grown well on plain agar without sealing the tubes, although better growths have been obtained by sealing. The last strain which was isolated in an atmosphere of increased CO_2 showed a good growth in 48 hours.

The positive identity of the organisms isolated was established by the agglutinin absorption test. The various strains were checked against known strains of Brucella abortus and anti-abortus serums. The cultures isolated from man could not be differentiated by serological methods from those recovered from cattle.

The first human strain which was isolated has proved to be slightly more virulent for guinea-pigs than the other four and has developed lesions similar to those described by men who have worked with the porcine strains. The other four are more virulent for guinea-pigs than the usual bovine type, but I have worked with several bovine strains that have shown just as marked virulence. As a rule the guinea-pigs injected with from 1 to 3 cc. of blood from the patients or with a small amount of the culture isolated from the blood, lost weight rather than increased in weight, as is customary with most of the bovine types. In several instances they became markedly emaciated. Cultures of Br. abortus extremely virulent for guinea-pigs were isolated daily from the milk drunk by the patient mentioned in the first case. Examination of the milk from the individual cows comprising this herd revealed that 50% of the animals harbored Brucella abortus in their udders and that the milk from two of the cows was heavily charged with strains of Brucella abortus more pathogenic for guinea-pigs than were the strains isolated from the other animals. Many of the guinea-pigs that were injected with the sediment and cream of mixed milk from this herd became greatly emaciated and died from 14 to 40 days after the injection. They showed extensive lesions of Brucella abortus infection and abscesses were invariably found in the epididymes and testes. In many cases the seminal vesicles were filled with pus. I have observed also extremely virulent bovine strains of Brucella abortus in other instances. I would like to mention one in particular isolated by K. F. Meyer [7] from certified milk. This culture was labelled by him "80" and in 1923 small amounts of this culture would kill guinea-pigs in three or four weeks. There were extensive lesions in the joints, reproductive organs, spleen and liver. Abscesses were present in the omentum and

[7] J. Infect. Dis., 1922, 31, p. 159.

lymph glands. One of my colleagues who has been working recently with this same culture has told me that it still shows marked virulence for guinea-pigs.

The intravenous injection of each of these human strains of Brucella abortus into pregnant heifers has been made. Five heifers in their first pregnancies were obtained from herds where no abortion had existed. Their blood serum was examined several times by the agglutination test for infectious abortion and found negative before the animals were given injections. Ten mils of washed agar cultures suspended in physiological salt solution were injected. Three of the suspensions gave a reading of 2 by the Gates [8] method of determining the density of suspensions, while the other two were slightly heavier and gave a reading of 1½. These five heifers aborted from 4 to 20 days after receiving the cultures. The organism has been recovered from the placenta and milk in each case. Four fetus gave pure cultures of the organism injected, but the fifth was expelled alive at approximately the eighth month of pregnancy. Inasmuch as we needed calves to nurse the dams he was not destroyed and cultured. Two of the cows became moribund from a pyometra and pyemia one month after abortion. They were destroyed and necropsied. Cultures of Br. abortus were obtained from the abscesses and uterus of each.

The injection of the same amount of Br. abortus from bovine sources by Birch and Gilman,[9] as well as other workers, has not produced abortion in such short periods of time as have cultures isolated from man. Nor have those bovine strains produced in animals reactions so marked as have the human strains. The bovine types do not always establish infection in the udder when cultures of the organism are injected intravenously. A significant fact in connection with these experiments is that in one cow the mammary gland became infected and Br. abortus was discharged continuously for 6½ months before she was destroyed. A calf which had nursed this cow became infected and, although it did not have any milk for four months after the dam was removed, it was still infected as evidenced by a high agglutinin titer and by the presence of Br. abortus in many of the lymph glands. In earlier work [10] on this problem of calf infection from milk, it was shown that as a rule Br. abortus cannot be recovered from the calf six weeks after the infected milk is removed.

[8] J. Exper. Med., 1920, 31, p. 105.
[9] Annual Report N. Y. State Vet. College at Cornell University, 1922-1923, p. 62.
[10] Cornell Veterinarian, 1924, 14, p. 16.

CONCLUSIONS

The first generation of cultures of Brucella abortus isolated from man was difficult to cultivate, but the second and following generations have grown well on nutrient agar in unsealed tubes.

The human strains have proven highly virulent for guinea-pigs but not more virulent than certain bovine strains with which the author has worked. Four of the strains have produced lesions in guinea-pigs which could not be distinguished from lesions produced by virulent types isolated from milk, placenta and bovine fetus.

Abortion was produced in five pregnant heifers by the intravenous injection respectively of five strains of human source. The organism was recovered from the colostral milk, placenta and fetus. The infection established itself in the udder and was present in the milk of one case for 6½ months after the animal aborted. The cultures isolated from man were more toxic for the pregnant heifers than the bovine types and produced abortion in a shorter time than have bovine strains as reported by other observers.

AGGLUTININS FOR BRUCELLA ABORTUS IN THE BLOOD OF MAN *

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Much evidence has been presented recently to show that certain strains of Brucella abortus may produce disease in man. For the most part the diagnosis of these infections has been accomplished by isolating the organism from the blood of these patients and by finding the specific agglutinin in the blood serum.

The agglutination test for infectious abortion in cattle has been used extensively since its value was demonstrated by Grinsted [1] and Holth.[2] It is not known just what percentage of infected animals does not show abortus agglutinins, but we are certain it is low.

Larson and Sedgwick [3] observed that in complement fixation tests of the serums from women who had aborted, the percentage containing abortus antibodies was greater than that giving positive Wassermann reactions. In studying the blood serums of 425 children they found by the agglutinin absorption test, specific abortus agglutinins in 17%. They showed that children who had drunk milk from a herd where infectious abortion did not exist, did not harbor any of these agglutinins, Nicoll and Pratt,[4] Cooledge,[5] and others have shown that specific abortus agglutinins were present in the serum of persons who had drunk raw milk containing Brucella abortus, while Nicolle, Burnett and Conseil [6] have demonstrated their presence in the serum of men injected with this organism. Evans [7] examined the blood serum of 500 patients suffering from various diseases and found that 59 contained specific agglutinins for Brucella abortus.

Keefer [8] reported the first case of Brucella abortus infection in America. The titer of the serum from this case was 1 : 3,200. Evans [7] reported finding abortus agglutinins in three serums, one of which had a titer of 1 : 160, while the other two had a titer of 1 : 320. Duncan [9] reported an abortus agglutinin titer of 1 : 2,400 with the serum of a patient suffering from undulant fever. Orpen,[10] DeKorte,[11] Ficai and Alessandrini [12] and Viviani [13] have reported similar cases which were undoubtedly due to Br. abortus. In these cases evidence pointed to infection from cattle. The blood serum from these patients had a fairly high titer.

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1 Maandsskr. fr. Drylaege, 1909, 21, p. 395.
2 Berl. Tierärtz. Wchnschr., 1909, 25, p. 686.
3 Am. J. Dis. Child., 1913, 6, p. 326.
4 Am. J. Dis. Child., 1915, 10, p. 203.
5 J. M. Research, 1916, 34, p. 459.
6 Compt. rend. Acad. d. Sci., 1923, 176, p. 1034.
7 Hygienic Lab., Bull. 143, 1925.
8 Bull. Johns Hopkins Hosp., 1924, 35, p. 6.
9 Brit. M. J., 1925, No. 3351, p. 554.
10 S. African M. Record, 1924, 22, p. 75.
11 Ibid., 1924, 22, p. 478.
12 Policlinico (sez.med.), 1925, 32, p. 113.
13 Ibid., p. 203.

During the last year I have studied 20 cases of undiagnosed fevers (table 1). Cultures were made and guinea-pigs were given injections with the blood and, in many instances, with the urine from each of the patients. Agglutination tests with the abortus antigen were made and with those serums in which agglutination occurred, except in case 15, agglutinin absorption tests were made to determine their specificity.

Brucella abortus was recovered in culture from five patients but agglutinins for this organism were present in only 3 of the 5. Specific abortus agglutinins were present in serum 13, but Brucella abortus was never recovered in culture from the blood. However, cultures for abortus were not made until after the symptoms had subsided. The

TABLE 1

RESULTS OF AGGLUTINATION TEST OF BLOOD SERUM OF 20 PATIENTS SHOWING SYMPTOMS RESEMBLING THOSE OF UNDULANT FEVER

No. of Case	Diagnosis	Bacteriological Results	Agglutination Tests with Brucella Abortus*
1	Undulant fever	Br. abortus	480
2	Not made	Staphylococcus albus	0
3	Not made	Negative	0
4	Subacute bacterial endocarditis.	Br. abortus	120
5	Not made	Negative	0
6	Not made	Negative	0
7	Undulant fever	Br. abortus	30
8	Typhoid fever	Br. abortus; Bact. typhosum	0
9	Typhoid fever	Bact. typhosum	0
10	Typhoid fever	Negative	0
11	Not made	Negative	0
12	Subacute bacterial endocarditis.	Streptococcus	0
13	Not made	Negative	30
14	Not made	Negative	0
15	Undulant fever	Unidentified Gram-negative rod.	240
16	Syphilis of lymph nodes	Br. abortus	0
17	Influenza	Negative	0
18	Not made	Negative	0
19	Acute articular rheumatism	Negative	0
20	Lagrippe	Negative	0

* The figures indicate the highest dilutions of serum producing agglutination; 0 indicates no agglutination with 1:5 dilution.

agglutinins did not appear in the blood serum until the end of the disease course which lasted approximately two months. Although the abortus antigen was agglutinated by serum 15, we did not have enough to complete an agglutinin absorption test. Patients 1 and 7, from whom Br. abortus was isolated in pure culture, had typical symptoms of undulant fever. Case 4, diagnosed by the clinician as subacute bacterial endocarditis, gave a culture of Br. abortus. This case was fatal and necropsy revealed an acute nephritis and marked splenomegaly.

Cases 8 and 16 are extremely interesting because abortus agglutinins were not present even in a 1 : 5 dilution of serum. In case 8 Bacterium typhosum was recovered in culture with Br. abortus and the serum

agglutinated typhoid bacilli in a dilution 1 : 60. The serum in case 16 gave a four plus Wassermann reaction and because of this and a general lymphadenitis, a diagnosis of syphilis of the lymph nodes was made by the clinicians in charge of the case. A lymph node was removed and sectioned. The examination revealed foci of infection similar in description to the lesions observed in lymph glands of guinea-pigs injected with abortus. The slides were not seen by the writer. It is difficult to interpret these findings and to know what significance should be given Br. abortus as a factor in producing disease in these two cases. In case 8 at the onset of the disease undulant fever was suspected by the physician in charge because of profuse night sweats and the absence of agglutinins for typhoid bacilli, and of rose spots. Later a more typical course of typhoid ensued and a satisfactory recovery was made. It is possible that the typhoid infection had produced an inflammation and perhaps ulceration of the intestine, facilitating the invasion of the blood by Brucella abortus.

It is not known whether abortus agglutinins are produced in serum with a positive Wassermann reaction. Many observers conclude that in multiple infections and immunization, the production at the same time of different agglutinins takes place without any change in the onset, intensity and duration. Hektoen,[14] on the other hand, believes that the normal development of agglutinins is altered in secondary infections. Lewis and Loomis [15] have shown that the allergic irritability is increased in guinea-pigs in the presence of tuberculosis. Schoenfeld and Carpenter [16] showed that guinea-pigs suffering from tuberculosis readily developed abortus agglutinins when they were infected subsequently with the latter organism.

The two cultures isolated in cases 8 and 16 produced abortion in susceptible pregnant heifers and appeared to be as virulent for them and for guinea-pigs as were the other three strains which had developed specific agglutinins in the blood serum of the patients from which they were isolated.

It has been observed frequently that the blood serum of an aborting cow will not show the presence of abortus agglutinins until a few weeks after the expulsion of the fetus. The placenta may show typical lesions of the disease and a culture of the organism can be recovered from it as well as from the tissues of the fetus.

[14] Harvey Lectures, 1909-1910, p. 163.
[15] J. Exper. Med., 1924, 40, p. 503.
[16] J. Infect. Dis., 1925, 37, p. 68.

A review of the literature on the agglutination test for diagnosing Malta fever shows that the titers of the serums from a large percentage of cases is from 1 : 10 to 1 : 100. There is a great difference of opinion among the various authors as to the titer of a serum that should be considered as conclusive evidence of Malta fever. Basset-Smith [17] favors a positive reaction in a dilution of 1 : 30, although he states that some cases of Malta fever occur where no agglutinins for Br. melitensis can be demonstrated. Other workers have had the same experience.

Inasmuch as Brucella abortus is at present considered only a variety of Brucella melitensis, it is to be expected that those strains which have been recovered from man will stimulate the production of agglutinins in the patient's serum in a manner and quantity not unlike melitensis. It is well known that certain strains of Brucella melitensis and Br. abortus make superior antigens while many others are unsatisfactory. This factor in itself may be responsible for variation occurring in the serum titer of many cases reported. We have used as an antigen in this work a suspension of living Brucella abortus 80, in physiological salt solution. The antigen gave a reading of 2.5 cm. on the Gates [18] apparatus for determining the density of suspensions. The readings were made after twelve hours' incubation. The technic of the agglutinin absorption tests is practically identical with that employed by Evans.[7] A heat-killed antigen was used with a density of approximately 48,000 p.p. M. after the serum which had been diluted 1 : 10 was added.

SUMMARY

The serums from 4 out of 20 patients showing symptoms suggestive of undulant fever contained specific agglutinins for Brucella abortus. The serum from a fifth patient who showed typical symptoms of recurrent or undulant fever agglutinated the abortus antigen completely in a dilution of 1 : 120. We were unable to obtain sufficient serum for an agglutinin absorption test.

Brucella abortus was isolated from the blood of five of the twenty patients. In the blood serums of two of these no abortus agglutinins could be demonstrated. However, one serum contained agglutinins for typhoid bacilli while the other gave a four plus Wassermann reaction. The titer of the serums varied from 1 : 30 to 1 : 480.

In the light of these findings it is evident that the agglutination test cannot be relied on to detect the presence of Br. abortus in man.

[17] Proc. Roy. Soc., 1906, pt. 4, p. 101.
[18] J. Exper. Med., 1920, 31, p. 105.

DISTRIBUTION OF HUMAN HEMOGLOBIN AND BEEF LENS PROTEIN FOLLOWING A SINGLE INTRAVENOUS INJECTION IN RABBITS

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The distribution of human hemoglobin and beef lens in rabbits after single intravenous injections has been studied. The rabbits were bled to death at different times after the injections, the various fluids saved and extracts made from the organs by grinding one gram of tissue with 1 or 2 cc. of salt solution and quartz sand; the mixtures were shaken, left in the ice-box over night, and the fluids drawn off after prolonged centrifugation. These extracts and other fluids were tested in progressive dilutions with strong hemoglobin and lens antiserums from rabbits. A resumé of the most significant results is given in table 1, in which the figures indicate the highest dilutions of the fluids that gave definite precipitates at the plane of contact with the serum after one hour at room temperature.

Human hemoglobin was given in pure solutions, in laked washed corpuscles, or in whole defibrinated or citrated blood. After injections of the citrated blood the hemoglobin was detected in the serum as late as 72 hours, and in the liver and bile up to 48 hours, but after injections of pure hemoglobin it failed of detection after 17 to 24 hours in both serum and organs. The lens protein was found frequently at 24 hours in a dilution of the serum 1 : 3 or 1 : 5 but not once at 48 hours.

Aside from this slight difference the two antigens were essentially alike in behavior. Both appeared within one-half hour in the urine, bile, intestinal mucosa and contents, and even in the froth about the nostrils of rabbits made dyspneic by injections of large amounts, thereby indicating not only a rapid general distribution but also the loss from the body of a considerable portion of antigen apparently unchanged.

In the organs the hemoglobin and lens content paralleled that of the serum but in lower concentrations, so there was a steady decrease from the first test after half an hour to a practically simultaneous disappearance from organs and blood without evidence of progressive accumulation or prolonged retention in any organ except as mentioned for hemoglobin

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after injections of whole blood. The kidney, marrow and omentum had the highest concentrations. The liver and mesenteric lymph nodes came next, and the spleen though comparable with the other organs in the early tests, gave only negative tests in 7 to 10 hours, an exception to the general gradual decrease already mentioned.

The urine content approached that of the serum and even exceeded it in a few instances due apparently to retention in the bladder, or in one instance to a general extravasation of hemoglobin into all tissues and fluids—synovial fluid 1 : 50, and urine 1 : 3200 (serum 1 : 400). The

TABLE 1

DISTRIBUTION OF HUMAN HEMOGLOBIN AND BEEF LENS PROTEIN FOLLOWING A SINGLE INTRAVENOUS INJECTION IN RABBITS

			Presence of Antigen in										
												Intestinal	
Antigens, Gm. per Kilo		Hours After Injection	Serum	Liver	Kidney	Spleen	Marrow	Omentum	Lymph Node	Bile	Urine	Mucosa	Contents
Hemoglobin.....	.4	1	500	...	...	150+	...	...	...	40	500+	...	...
Hemoglobin.....	.4	2	1000+	200	800	100	100	400	50	...	160	...	...
Hemoglobin.....	.5	10	400	10	100	0	100	100	10	...	3200	...	...
Hemoglobin.....	.15	20	10	20	0	0	0	0	...	+	...	0	0
Hemoglobin.....	.15 to .3	22-48	0*	0*	0	0	0	0	0	0*	0	...	...
Beef lens.....	.13	½	400	160	320	160	160	6+	320	160	...	160	160+
Beef lens.....	.25	1	1000+	100	100	...	...	...	...	...	100	...	...
Beef lens.....	.25	7	100+	16	32	0	50+	50	10	5	150	2	0
Beef lens.....	.25	17	50	5	5	2	5	10	5	10	10	...	...
Beef lens.....	.25	48	0†	0	0	0	0	0	0	0	0	0	0

* Hemoglobin present after injection of citrated blood, (.3 gm. per kilo estimated).
† Lens present in serums 24 hours after injection.

appearance of dark, well defined streaks in the kidney was usual after injections of hemoglobin. The relations between the liver and bile were less regular than those of the kidney and urine probably because so small an amount of bile, 2% of the daily secretion in rabbits enters the gallbladder,[1] and the hourly secretion averages 7 to 8 cc. as collected from a fistula. One collection therefore at the time of killing an animal affords only partial information. In the rabbit the bile from the gallbladder is strongly alkaline, and even when neutralized it forms precipitates with protein solutions including egg white,[2] dog serum and even rabbit serum occasionally, so careful controls are required.

Studies of the distribution of cellular antigens are numerous. Wyssokowitsch[3] injected yeasts, bacteria and spores, intravenously into rabbits and dogs and by subsequent cultures found the blood soon sterile, the liver containing large numbers

[1] Neilson and Meyer: J. Infect. Dis., 1921, 28, p. 511.
[2] Kimura, N.: Jap. M. World, 1923, 3, p. 207.
[3] Ztschr. f. Hyg. u. Infektionskrankh., 1886, 1, p. 1.

of cultivable organisms, the marrow, kidneys, lungs and lymph nodes with varying content, while the bile and urine were generally sterile unless lesions of the liver and kidneys were present. Similar results emphasizing the early disappearance from the blood, the prevailing high contents of the liver and spleen, and the varying importance of the other organs, were also reported by Werigo[4] from histologic examination of rabbits' tissues after intrajugular injections of anthrax bacilli; by Bail[5] with cultures from both rabbits and guinea-pigs after intravenous and intrapleural injections of cholera and typhoid organisms; by Arima,[6] Bull,[7] Parker and Franke,[8] Stone[9] and Meyer Neilson and Feusier[10] also by cultures after intravenous injections of typhoid bacilli in rabbits; in Kyes'[11] observations of the phagocytosis of pneumococci by the hemophages of the pigeon; by Bartlett and Ozaka[12] working with killed Micrococcus aureus in dogs; and by Nagao[13] in a detailed study of the cellular changes in guinea-pigs after intrajugular or intraventricular (left heart) injections of killed nonhemolytic streptococci. Many of these authors[4, 6, 12, 13] and others[14] have observed the accumulation of bacteria in the lung immediately after injection, probably largely from mechanical influences; the phagocytosis there chiefly by polymorphonuclear leukocytes; and the distribution later, within 30 minutes to 1 hour,[13] to other organs. Intraperitoneal injections of typhoid bacilli by Buxton and Torrey,[15] and of pneumococci in pigeons by Berry and Melick,[16] also gave similar results with the special features of a delayed (about 2 hours) general distribution, and the local accumulation of organisms both free and within macrophages on and within the omentum.

The mechanism of gallbladder infections, whether by the duct from the intestine, from the portal system through the liver cells, or from emboli in the vesical wall has been reviewed by Meyer, Neilson and Feusier,[17] who agree with Carmichael and Else that infection through the portal system is not easily obtained, and support Chirolanza's[18] earlier belief that the organisms enter the bile from emboli in the wall with direct extension through the tissue. There seems, however, to be considerable evidence that the organisms do traverse injured liver tissue, and there is perhaps an important suggestion concerning this question in the statement that gallbladder infections constantly follow intrasplenic injections of typhoid bacilli,[17] when correlated with the observation[19] that lesions of the liver follow injury of the spleen by crushing.

Cells other than bacterial have also been observed within these organs after injections by different routes. Cantacuzene[20] described the resorption of hepatic cells after intraperitoneal and intravenous injections. The macrophages of the omentum, liver and spleen were especially involved. Spermatozoa from guinea-pigs and red blood cells from the goose, injected intraperitoneally into guinea-pigs (Metchnikoff[21]), were taken up by macrophages with slender protoplasmic pro-

[4] Ann. de l'Inst. Pasteur, 1894, 8, p. 1.
[5] Arch. f. Hyg., 1905, 52, p. 272.
[6] Ibid., 1911, 73, p. 265.
[7] J. Exper. Med., 1916, 23, p. 419.
[8] J. Med. Research, 1919, 39, p. 301.
[9] J. Infect. Dis., 1919, 25, p. 284.
[10] Ibid., 1921, 28, p. 408.
[11] Ibid., 1916, 18, p. 277.
[12] J. Med. Research, 1917, 35, p. 465.
[13] J. Infect. Dis., 1920, 27, p. 327.
[14] Ito, H.: J. Med. Research, 1917, 37, p. 189. Hopkins, J. G., and Parker, J. T.: J. Exper. Med., 1918, 27, p. 1.
[15] J. Med. Research, 1906, 15, pp. 5, 18.
[16] J. Immunol., 1, p. 119.
[17] J. Infect. Dis., 1921, 28, p. 456.
[18] Ztschr. f. Hyg. u. Infektionskrankh., 1909, 62, p. 11.
[19] Fuessinger, Ravina, and Javins: Rev. de Med., 1925, 42, p. 457.
[20] Ann. de l'Inst. Pasteur, 1902, 16, p. 522.
[21] Ibid., 1899, 3, p. 737.

longations and finally lodged, some locally within the fibrinous coating on the omentum particularly over the milky spots, some within the omentum and some carried by the lymph and blood to the liver, spleen and lymph nodes. This early description of the mononuclear phogocytosis notes also the minor activity of the polymorphonuclear leukocytes. Portis [22] described the phagocytosis of sheep red cells by clasmatocytes of the omentum after intraperitoneal injections but he does not at the same time compare the content of this organ with that of others. It will be noted that the omentum has been studied in connection with intraperitoneal injections but otherwise generally omitted. Kyes' [23] recognition of the rôle of the fixed tissue phagocytosis of red blood cells in the liver and spleen of the pigeon has been followed by studies by Cary [24] and Motohashi [25] on the disposal of foreign blood cells in the rabbit. Motohashi considers the active hemophages of the spleen the most important cells and the potential hemophages of the spleen, liver and bone marrow as assuming importance with large injections or other conditions not adequately met by the normally active cells. This compensatory action of the liver, lymph node and especially of the marrow is also described by Peabody and Broun [26] for certain diseases including pernicious anemia.

On the evidence of antibody response, antigens are removed from the tissue at the site of injection (rabbit ear, intracutaneous injections), within one-half hour,[27] and the transfer of antigen is accomplished only when resorption into the blood stream is possible, according to Cohn.[28] After intramuscular injections of horse serum in partly amputated legs of guinea-pigs followed by severance of the connecting tissue (isolated skin, muscle, bone, artery or vein) within two minutes, he found that sensitization resulted only when the vein (femoral) had been the connection. The disappearance of red cells (goat), at least of their antigenic properties, from the blood of dogs within a few hours after injections, was demonstrated by Hektoen and Carlson [29] in the failure of dogs, which were recipients of blood from the treated dogs, to produce lysins, agglutinins and opsonins for the red cells. Injections of emulsified organs instead of blood transfusions in similar experiments (Luckhardt and Becht [30]), indicated by the response of the recipients that the spleen contained more antigen from the red cells 24 hours after injection than the marrow, lymph nodes, liver or heart muscle.

The distribution of noncellular antigens in the organs has not been so generally studied. In transfusion experiments similar to those described,[29] horse serum injected into dogs, soon lost its precipitogenic power.[31] This is not in accord with the demonstrations by precipitin tests that at least some reacting part of the injected horse serum remains in the rabbit blood serum as late as the seventh or ninth day,[32] although precipitins for whole horse serum may appear by the fifth or the seventh day.

After injections of human whole blood into chickens we also obtained positive precipitin tests of the organ extracts with laked human blood antiserum as late as the seventh day. The spleen, marrow, liver, lung and

[22] J. Infect. Dis., 1923, 33, p. 159.
[23] Monatschr. f. Anat. u. Physiol., 1914, 31, p. 543.
[24] J. Infect. Dis., 1915, 17, p. 432.
[25] J. Med. Research, 1922, 43, p. 419.
[26] Am. J. Path., 1925, 1, p. 169.
[27] Friedberger and Tinti: Ztschr. f. Immunitätsforsch., 1924, 39, p. 452. Friedberger and Huang: Ibid., p. 459. Friedberger and Torii: Ibid., p. 462.
[28] Ztschr. f. Hyg. u. Infektionskrankh., 1926, 106, p. 209.
[29] J. Infect. Dis., 1910, 7, p. 319.
[30] Am. J. Physiol., 1911, 28, p. 257.
[31] Pettit and Carlson: J. Infect. Dis., 1912, 10, p. 43.
[32] Gay and Rusk: Univ. Calif. Public Path., 1912, 2, p. 59.

kidney were the most important after intramuscular injections, but in the more irregular results following intraperitoneal injections the liver, lung, and serum were generally negative.

The presence of antigen in the blood after precipitins have appeared has been observed by many investigators [33] and explained by the protective action of colloids, by the union of antigen and antibody according to the laws for mass action, and by the nonhomologous relation of these substances due to the presence in serums, eggwhite and such antigens of many individual antigenic fractions whose absorptions are completed at different times, so that the appearance of some of the corresponding precipitins, soon after the injection overlaps the period of absorption for the other antigenic fractions. The use of single antigens [33] strongly supports this latter explanation although there are some contrary reports.[34] It might well be mentioned that precipitin for human hemoglobin after injections of pure solutions did not appear before the disappearance of the antigen according to precipitin tests with antihuman hemoglobin rabbit serum.

Proteins are excreted in the urine when ingested in large amounts,[35] or injected, even intracutaneously,[36] although there is evidence that some solutions are more pervasive than others—eggwhite than blood serum, purified albumin than whole eggwhite (Wells [35]). The dispersion of the colloid or the size of the molecules or particles seems to have an important influence on the excretion and probably on the disposal of the injected antigens.[37]

The results which most nearly correspond to ours are those of Vaughan, Cumming and McGlumphy [38] who, in their work with eggwhite in rabbits, found by sensitization tests in guinea-pigs that whether given by stomach, by rectum, or intra-abdominally, the antigen appeared in the blood, and when given intravenously it disappeared from the blood in about 1½ hours but could still be detected for a short time in the bile, urine, and organs. The kidneys, spleen, brain and muscle seemed to contain more antigen than the liver but after transfusion of the animal the fluid contained antigen, the kidney and brain had lost it, while the liver, spleen and muscle were positive. The interpretation was that the egg white was loosely held in the brain and kidneys, and perhaps rapidly fixed in the liver. Gay and Rusk [32] have pointed out that although precipitin tests with antiserum for horse serum can detect horse serum in the blood of the rabbit for many days after its injection that tests for sensitization to horse serum in guinea-pigs given injections with the blood of such rabbits are negative after the first 24 hours. Whether the difference is due to the differences of the two methods, or to a difference between the stimulating and the reacting parts of the antigen cannot be

[33] Opie, E.: J. Immunol., 1923, 8, p. 19.
[34] Bayne-Jones, S.: J. Exper. Med., 1917, 25, p. 851.
[35] Ascoli, A.: München. med. Wchnschr., 1902, 26, p. 734. Wells, H. G.: J. A. M. A., 1909, 63, p. 863.
[36] Opie, E.: J. Exper. Med., 1924, 39, p. 660.
[37] Oka, Toru: Tohoku J. Exper. Med., 1926, 7, p. 94. Drinker, Shaw and Drinker: J. Exper. Med., 1923, 37, p. 829.
[38] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1911, 9, p. 16.

stated with certainty. Since single antigens are also anaphylactogenic the presence of many antigenic fractions in the serum does not answer the question.

The distribution of solutions of antigens therefore differs somewhat from that of cellular antigens at least in the few available quantitative studies. The liver and spleen which share the most important places in containing cultivable bacteria and red blood cells are prone to give early negative tests with nonparticulate antigens while the kidneys in both reports (Vaughan, Cumming, and McGlumphy and ours), and the omentum and marrow in our work, have the higher content and the other organs are of less and more varying importance. After intraperitoneal injections of cellular antigens the macrophages both within the omentum and on its surface were shown to be actively phagocytic.

Although differing in the order of importance the organs containing formed or cellular antigens, and those containing nonparticulate antigen in highest concentrations after intravenous injections, form the same group. This is also the same group of organs which contains in normal conditions the greatest numbers of the phagocytic cells named collectively by Aschoff and Landau (Aschoff),[39] the reticulo-endothelial system. The possibility of variations of the state of receptivity[40] of different sets of cells in this system suggests at once a reason for varying results with different antigens, with different modes of injections, and with individual animals. The changes due to compensatory action, the transition of nonphagocytic to phagocytic cells, and the mobilization of phagocytic cells by the blood and lymph, are also factors of influence.

SUMMARY

The two antigens, human hemoglobin and beef lens, as detected by antiserums after intravenous injections in rabbits, had similar distributions. Both disappeared in about 24 hours depending on the amount injected; the lens substance perhaps a trifle more slowly than the hemoglobin. Hemoglobin persisted longer after injections of whole blood than after injections of pure solutions.

A considerable quantity of antigen was found unchanged in the urine, bile, and intestine soon after injection. The relations between the liver and bile were less regular than between the kidney and urine, probably because of the manner of collection and of excretion and storage in the respective bladders.

[39] Ergebn. inn. Med. u. Kinderheilk., 1924, 69, p. 1352.
[40] Krumbhaar, E. B.: Internat. Clinics, 1925, 35, 2, p. 280.

The antigens were in highest concentrations in the marrow, omentum and kidneys, but seemed to be present to some extent in many tissues. The spleen became negative sooner than any other organ. After the first test one half hour after injection there was no sign of accumulation, of selective absorption, or of prolonged retention in any organ after the disappearance from the blood.

The results with these solutions do not differ essentiallly from those described by others with both cellular and noncellular antigens, and the organs shown to have high concentrations of these antigens after intravenous injections are the organs that in general are known to be best equipped with cells of the reticulo-endothelial system.

INFLUENCE OF HIGH PARTIAL PRESSURES OF OXYGEN ON THE GROWTH OF CERTAIN MOLDS

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It is of interest that a gas so necessary to maintenance of life may, in concentrations well beyond the normal of the atmosphere, be injurious.

It is well known that in high concentrations, oxygen is injurious to the lungs of mammalia.[1] In contrast, the work of Cleveland,[2] indicates that certain invertebrates and amphibia apparently are unharmed by high oxygen concentrations. Protozoa are injured and ultimately killed by high oxygen concentrations, apparently regardless of whether they are parasitic or free living organisms (Cleveland[2]). Some bacteria are inhibited in growth and others are not. Although not uniformly so, this effect seems more pronounced on parasitic than on saprophytic bacteria.[3] The human tubercle bacillus is definitely inhibited in growth, where the oxygen concentration is 80 per cent or higher, and exhibit decreased respiration (Novy and Soule[4]). Previously, Moore and Williams,[5] and Adams[3] had shown the same effects on avian tubercle bacilli. Although Moore and Williams found that high concentrations of oxygen kill tubercle bacilli, the other studies noted indicate that the effect is merely inhibitory. This was true of our studies with other bacteria.

The effect on cultures of the cells of vertebrates is also variable. Burrows[6] found that cultures of chicken embryo heart and skin are not inhibited. Fischer and Anderson[7] report that high concentrations of oxygen modify the metabolism, inhibit the growth of, and even kill the cells of the Rous chicken sarcoma, whereas similarly treated cultures of fibroblasts are much less susceptible.

As a result of our studies on bacteria, it was thought wise to determine the influence of high partial pressures of oxygen on higher vegetable organisms and accordingly certain cultures of molds were obtained. For the plant molds we are indebted to Dr. C. L. Shear of the U. S. Department of Agriculture.

The organisms were grown first on Sabouraud's medium (water 1,000 cc., peptone 10 Gm., glucose 10 Gm., and agar 20 Gm.) slanted in test tubes. For the comparison of growth in air and in high oxygen concentrations, a small section of agar and growing mold, about 5 mm. in diameter was cut out of the agar slant. This was divided into two approximately equal parts. Each section was transferred to

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[1] Karsner, H. T.: J. Exper. Med., 1916, 23, p. 149.
[2] Biol. Bull., 1925, 48, p. 455.
[3] Karsner; Brittingham, and Richardson: J. M. Research, 1923, 44, p. 83. Adams, A.: Biochem. J., 1912, 6, p. 297.
[4] J. Infect. Dis., 1925, 36, p. 168.
[5] Biochem. J., 1909, 4, p. 177; 1911, 5, p. 181.
[6] Am. J. Physiol., 1917, 43, p. 13.
[7] Ztschr. f. Krebsforsch., 1926, 23, p. 12.

Petri dish plates of the Sabouraud medium, one to be grown in air and the other in oxygen. The Petri dishes, with earthenware covers, were stacked in glass jars.

For cultivation, 2 liter glass jars, with a layer of soda lime in the bottom, were employed. The jar tops, provided with two glass stoppered tubes, were sealed to the jars by means of vaseline. One tube ran to the bottom of the jar. Both tube outlets were open and into one was forced oxygen under pressure and into the other air under approximately equal pressure. Several jets were run in and then both stopcocks were closed. After allowing a few minutes for diffusion, distilled water was run into the bottom of the jar through the longer tube so as to displace enough gas through the other tube for analysis. Analysis was made at the beginning and the end of the experiment. Sealing was excellent and the variations slight, but in the tables only the last analysis is given. For the lower percentages of oxygen the mixture was made in a gasometer tank and forced into the jars by hand pressure on the top of the tank. The oxygen was commercial gas prepared from air by the Linde Air Products Company and was determined to be 99% oxygen.

After performing the experiment given in table 1, the injection of air in the control jar was abandoned. It was suspected that the pressure in the oxygen jar might be for a time slightly above barometric pressure, but when it was found that the molds grew at the same rate in the jars

TABLE 1

COMPARATIVE SIZES OF MOLD COLONIES IN NATURAL AND INJECTED AIR

Mold	Percentage of Oxygen	Growth in Days	Measurements in Cm. in Two Diameters	
			Air Injected	Air Natural
Microsporon lanosum	96	4	1.2 × 1.5	1.6 × 1.6
Trichophyton niveum radians	96	4	2.7 × 1.6	2.4 × 1.7
Trichophyton gypseum asteroides	96	4	1.0 × 1.0	1.2 × 1.0
Epidermophyton inguinale	96	4	2.1 × 2.0	2.3 × 1.8

where air was injected as in those where it was not, it was decided to omit the more exact duplication of method in the air jars.

The cultures were incubated at room temperature until the colonies were large enough to permit of reasonably accurate measurements.

In those few instances where yeasts were studied, the method of planting was the same as that described in a previous paper on bacteria (Karsner [3]).

The molds and yeasts employed are given in the following list:

Microsporon lanosum, Trichophyton fumatum, Trichophyton niveum radians, Trichophyton gypseum asteroides, Epidermophyton inguinale, Trichophyton gypseum lacticolor, Achorion schoenleinii, Trichophyton accuminatum, Sporotrichum, Mastigocladium B, Trichophyton crateriforme, Trichophyton purpureum, Saprolegnia from flies (not further identified), Pencillium glaucum (not further identified), Air molds (a) pigmented, (b) not pigmented, (not identified).

Except as noted, the cultures are pure. Nevertheless, it is not certain that all are free from bacterial contamination, for in some sent to Dr. Margaret Church of the U. S. Department of Agriculture, such contamination was reported in two instances.

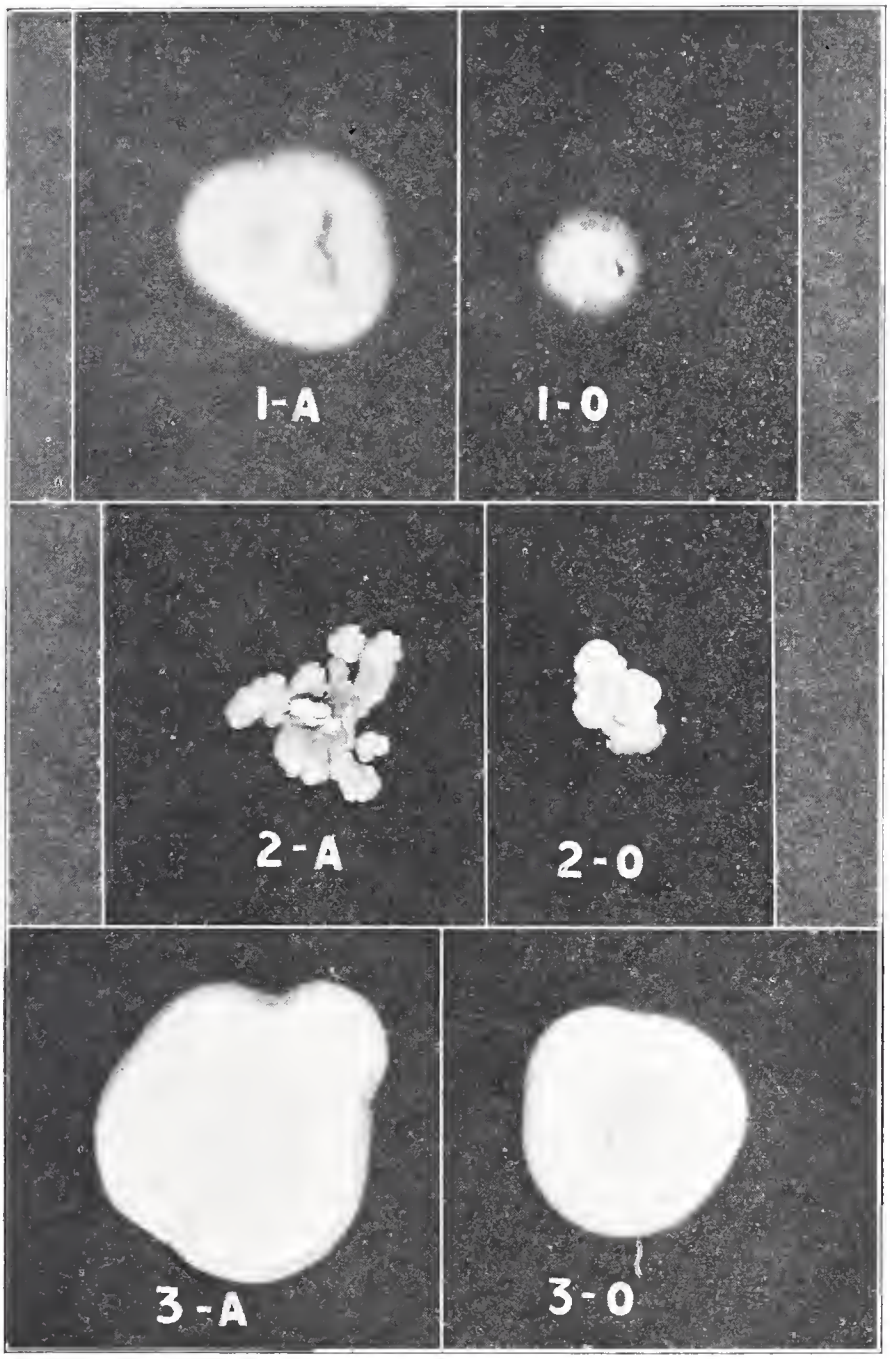

Fig. 1.—Colonies (actual size) of Epidermophyton inguinale (1), Monilia candida (2), and Microsporon lanosum (3) grown in air, A, and high concentrations of oxygen, O, for the same period of time.

TABLE 2

SIZE OF MOLD COLONIES AT DIFFERENT OXYGEN PRESSURES

Percentage of Oxygen	Type of Mold	Growth in Days	Measurements in Cm. in Two Diameters: In Air	Measurements in Cm. in Two Diameters: In Oxygen
50	Microsporon lanosum	5	2.0 × 1.9	2.0 × 2.0
	Trichophyton fumatum	5	1.8 × 2.0	1.8 × 2.0
	Trichophyton gypseum asteroides	5	2.1 × 3.0	2.0 × 3.0
	Trichophyton crateriforme	5	4.0 × 4.0	4.0 × 4.0
60	Trichophyton niveum radians	5	3.9 × —3.6	3.8 × 3.6
	Epidermophyton inguinale	5	4.3 × —4.0	4.3 × 4.0
	Sporotrichum	5	2.2 × 2.1	2.2 × 2.0
	Trichophyton purpureum	5	2.5 × 2.0	2.5 × 2.0
70	Trichophyton niveum radians	4	2.0 × 1.8	2.0 × 1.9
	Epidermophyton inguinale	4	2.3 × 2.4	2.2 × 2.5
76	Trichophyton niveum radians	4	1.5 × 2.0	1.5 × 1.2
	Epidermophyton inguinale	4	1.8 × 1.0	1.5 × 1.0
	Sporotrichum	4	2.0 × 2.0	2.0 × 1.2
89	Sporotrichum	4	2.3 × 2.6	2.1 × 1.6
	Trichophyton niveum radians	4	2.5 × 1.5	1.5 × 1.9
90	Microsporon lanosum	4	2.5 × 1.0	2.1 × 0.5
	Trichophyton fumatum	4	2.5 × 0.4	1.9 × 0.9
	Trichophyton niveum radians	4	5.5 × 6.0	2.5 × 2.5
	Achorion schoenleinii	4	1.5 × 1.4	0.9 × 0.4
	Trichophyton accuminatum	5	2.5 × 3.0	2.1 × 2.1
	Mastigocladium B	5	2.8 × 2.2	1.8 × 2.0
	Microsporon lanosum	5	1.8 × 1.6	1.0 × 1.3
	Trichophyton purpureum	5	2.2 × 2.8	2.0 × 2.5
	Trichophyton crateriforme	5	2.8 × 3.0	2.3 × 2.6
92	Trichophyton niveum radians	2.5	1.5 × 1.5	1.5 × 0.5
	Trichophyton accuminatum	2.5	1.4 × 0.9	0.6 × 0.3
	Trichophyton fumatum	2.5	1.2 × 0.7	1.0 × 0.6
	Trichophyton gypseum lacticolor	2.5	0.8 × 0.6	0.6 × 0.4
94	Epidermophyton inguinale	3	2.0 × 1.7	1.5 × 1.2
	Trichophyton niveum radians	8	3.2 × 2.2	2.0 × 1.6
	Epidermophyton inguinale	8	3.0 × 2.3	1.8 × 1.2
95	Microsporon lanosum	5	3.0 × 2.0	1.5 × 0.9
	Trichophyton fumatum	5	2.8 × 2.7	2.0 × 1.7
	Trichophyton niveum radians	5	6.0 × 6.5	3.4 × 2.6
	Trichophyton gypseum asteroides	5	2.3 × 2.2	2.7 × 1.6
98	Microsporon lanosum	5	2.3 × 1.5	1.2 × 1.5
	Epidermophyton inguinale	5	5.2 × 3.0	2.1 × 2.0
	Trichophyton niveum radians	5	5.5 × 3.2	1.8 × 2.0
	Trichophyton crateriforme	5	2.7 × 2.0	1.6 × 1.4
99	Trichophyton niveum radians	5	3.5 × 3.5	1.5 × 1.8
	Epidermophyton inguinale	5	4.0 × 4.0	2.0 × 1.8
	Trichophyton accuminatum	5	3.0 × 3.5	2.3 × 2.5
	Sporotrichum	5	2.5 × 3.0	1.8 × 2.4
89	Air molds	4	4.5 × 4.5	6.0 × 6.0
		4	4.3 × 4.0	5.5 × 5.0
90	Air molds	4	5.5 × 3.0	5.5 × 7.5
92	Air molds	4	2.0 × 4.0	7.0 × 2.5
		4	5.0 × 3.5	3.5 × 3.0
94	Air molds	3	4.5 × 4.0	4.0 × 3.0
		3	4.8 × 2.6	4.0 × 2.3
89	Air molds	3	2.7 × 2.0	2.4 × 1.5

The plates inoculated with Saprolegnia from flies and with the specimen of Penicillium glaucum showed no demonstrable differences in any concentration of oxygen.

Examination of table 2 shows that the common air molds are not inhibited in growth by the oxygen. The molds which have parasitic capacity show no inhibition by oxygen concentrations of 50, 60 and 70%. At a concentration of 76%, inhibition is apparent. At percentages of 89 and more, the inhibition is generally apparent and in many instances marked. As with the lungs of mammals, and bacteria, including tubercle bacillus, a concentration in the neighborhood of 80% seems to be the critical point beyond which injurious effects are undoubted.

Following this study, the organism of Madura foot, isolated by Gammel, Miskdjian and Thatcher,[8] was treated in the same manner and

TABLE 3

PLANT MOLDS IN OXYGEN CONCENTRATIONS, 96 TO 98%

Name of Mold	Growth in Days	Diameters in Cm. In Air	Diameters in Cm. In Oxygen
Sclerotinia fruticola (Wint)	3	2.0 × 1.8	0.5 × 0.5
Monilia candida (Berkhout)	3	2.3 × 2.0	1.5 × 1.3
Cryptococcus (Souche Buschki)	3	1.5 × 2.0	1.0 × 1.1
Cryptococcus hominis	3	2.2 × 2.0	2.2 × 2.0
Allescheria boydii (Shear)	3	1.0 × 1.0	1.0 × 1.0
Aspergillus jeanselmei*	3	2.0 × 2.3	2.2 × 2.0
Alternaria (Wahl)	3	2.5 × 2.5	2.5 × 2.5
Dematium chodati (Candida chodati, Berkhout)	3	2.2 × 2.1	1.9 × 2.1
Oidium suaveolens var. minuta	3	3.0 × 3.0	2.8 × 3.0

* Confirmation of the exact identity of this culture is lacking.

in two experiments with oxygen concentrations of 96 and 97%, the air colonies were respectively 0.7 x 0.8 cm., and 0.8 x 0.8 cm., whereas the corresponding oxygen colonies were 0.4 x 0.5 cm., and 0.7 x 0.5 cm.

A series of plant molds were studied, as in table 3.

Examination of table 3 comprising organisms capable of plant parasitism, with the possible exception of Aspergillus jeanselmei, shows that only three are inhibited by oxygen concentrations of 96 to 98%. Inhibition does not seem to bear a fixed relationship to parasitism.

In all studies of pigmented molds, careful comparison was made of the depth and quality of the color in air and in oxygen. In the work with bacteria there was an inhibition of pigment production but in the molds no difference was discernible.

[8] Arch. Dermat. & Syph., 1926, 13, p. 66.

CONCLUSIONS

Concentrations of oxygen of 76% and more, exercise a definite inhibitory effect on certain molds. In a general way this is most apparent in molds with the capacity for parasitism. It is not a fixed corollary of parasitism, because some of the parasitic molds, more especially the plant parasites, show no inhibition.

There is no fixed relation between the degree of inhibition and the percentage of oxygen concentration, although the higher percentages appear to be more certain in effect. The inhibitory effect is apparent from the time growth of the colony starts until the end of the observation. In no instance was the organism killed by the oxygen percentages employed and on removal from the oxygen chamber growth appeared to progress at a normal rate.

The production of pigment by the molds is not altered by growth in any of the oxygen concentrations used.

DRUG-FASTNESS IN ITS RELATION TO THE RESISTANCE OF CERTAIN ORGANISMS TOWARD FAMILIAR GERMICIDES

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The various factors which lead to the death of bacteria by chemical agents have long been a subject of intensive investigation. In general these studies have been directed along two lines. One group deals with the measurement of the activity of a given disinfectant when compared with a recognized standard, such as phenol. The other consists mainly of attempts to find disinfecting agents which are sufficiently powerful to destroy bacteria in the tissue without themselves being toxic. In the course of certain investigations conducted in this laboratory a series of observations on patients were made which seemed to indicate that at least some strains of bacteria may become fast or resistant to the drug used in the treatment of pathological conditions. If bacteria can be made to become drug fast an explanation for certain well recognized clinical facts may be found.

The literature contains only a few references to experimental work in which an attempt has been made to produce any considerable degree of drug fastness in bacteria. A few strains of bacteria have been developed whose resistance to the disinfecting action of a given germicide has been increased to a slight degree.

Abbott[1] developed some strains of pyogenic cocci which would grow after exposure to a strength of 1 : 1,000 bichloride of mercury for 20 minutes although the original culture failed to grow after exposure for more than five minutes. Altman and Rauth[2] were able to produce a strain of Bacillus coli which was drug fast to phenol, but they gave no details as to the initial and final strength of the disinfectant. Marks[3] observed an eight-fold rise in the resistance of a strain of hog-cholera bacillus to arsenious acid by subculture in mediums containing increasing strengths of the acid. The resistance of this strain to antimony was found to have been increased forty-fold by this treatment. Regenstein[4] cultivated Staphylococcus aureus, Bacillus coli and Bacillus typhosus in broth to which had been added concentrations of a variety of germicides which had originally pre-

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[1] Bull. Johns Hopkins Hosp., 1891, 2, p. 50; J. M. Res., 1912, 21, p. 513.
[2] Ztschr. f. Immunitätsforsch. u. exper. Therap., O., 1910, 7, p. 629.
[3] Ibid., 1910, 6, p. 293.
[4] Centralbl. f. Bakteriol., I, O., 1912, 63, p. 281.

vented growth of these cultures. Ehrlich [5] and his co-workers and also Browning [6] noted that in some instances infections treated with arsenicals did not respond in the usual manner but proved to be extremely resistant to specific drug therapy. He attributed this to an increased tolerance or drug fastness of the organism. The idea of complete disinfection by means of a massive dose of the drug in question, which he designated as "therapia sterilisans magna," was based on this observation. His theory of drug fastness was its direct result. In view of Ehrlich's experimental observations it is interesting to note that Gonder [7] reported the development of arsenic fast strains of Spironema gallinarum in vitro. Although Dallinger [8] did not work with drugs, he developed strains of protozoa with a greatly increased tolerance toward heat. The optimum temperature for growth of these organisms was 60 F., but by frequent cultivation at increasing temperatures the protozoa were, after seven years, successfully grown at 158 F. In all this work the importance of the process of selection must be borne in mind.

Most of the work relative to the production of strains of microorganisms showing increased resistance to a given therapeutic agent such as Ehrlich observed in arsenic-fast strains of trypanosomes, has been complicated by necessity of considering the part played by the tissues of the host in the phenomenon. Although the mechanism by which the drug fast character is acquired by an organism infecting the blood stream or tissues is still very imperfectly understood—it seems clear that the tissues of the host play some essential rôle. If, on the other hand, we consider only surface infections, in this connection, such as infections of the urinary tract, in which the organisms may be exposed to the therapeutic agent either through its excretion into the urine or by direct application to the mucosa, we are dealing with conditions which are fundamentally different and far simpler—conditions which more nearly approach those in the test tube. For this reason a comprehensive series of experiments designed to determine whether certain organisms commonly found in urinary infections could be made more resistant by exposure to the germicides and internal urinary antiseptics ordinarily employed clinically, appeared to be of considerable interest.

The problem was undertaken with four general ideas in mind; to determine whether bacteria become drug fast in vitro; whether this drug fastness, if developed, is specific; whether a drug fast strain is more susceptible to the action of other germicides; and finally, to ascertain how long the acquired character persists when the drug fast organisms are cultivated in an environment free from the germicide to which they are fast.

[5] Berl. klin. Wchnschr., 1907, 44, p. 9.
[6] Brit. M. J., 1907, 2, p. 1405.
[7] Centralbl. f. Bakteriol., I, O., 1912, 62, p. 168.
[8] J. Roy. Microsc. Soc., 1887, 1, p. 185.

Cultures.—Five organisms were selected for study. Cultures A, the Hopkins strain of Bacillus typhosus, was selected because it is used as the standard organism for testing disinfectants by the U. S. Hygiene Laboratory method and served to a certain extent as a control on the activity of the germicides used. Culture B was a strain of Bacillus coli isolated from the stool of a typhoid carrier who had been taking hexylresorcinol for one year. This strain was selected because there was the remote possibility that long continued use of a germicide taken into the digestive canal might have produced a certain degree of drug fastness in the intestinal flora. Culture C was a strain of Bacillus coli isolated from the stool of an individual who had not been under treatment with any drug. Culture D was a strain of Bacillus lactis aerogenous isolated from the urine of a patient with a urinary infection caused by this organism. The patient had been under treatment with hexylresorcinol for over one year. Here again there is the possibility that the long continued use of a urinary antiseptic may have increased the resistance of this strain to the drug. Culture E was a strain of Bacillus coli isolated from a mixed urinary infection with Bacillus pyocaneus. No drug had been administered prior to the collection of samples.

Germicides.—The germicides used in this investigation are those commonly employed in the treatment of urinary infections and can be conveniently divided into two groups: those which are applied directly to the mucous surfaces and those which are administered orally and excreted in the urine. The former group includes silver nitrate and mercurochrome; while formaldehyde, which is produced in acid urine from urotropin and hexylresorcinol fall into the latter group. Neutral acriflavin is employed by both methods and is generally considered to be an antiseptic rather than a germicide. Finally, phenol was included because of its general use as a standard in testing distinfectants. All were of the usual medicinal degree of purity.

Technic.—Agar slants, containing various dilutions of each germicide were prepared as follows: Standard nutrient agar was sterilized in flasks containing about 125 cc. This medium was transferred aseptically by volumetric pipettes in 4 cc. amounts to sterile test tubes and incubated 24 hours to insure sterility. Accurate dilutions of aqueous solutions of the several germicides were prepared so that 1 cc. added to 4 cc. of melted agar would give the desired final dilution. The tubes were slanted and after solidification again incubated to further insure sterility.

Immediately after isolation the strains of Bacillus coli and Bacillus lactis aerogenes together with Bacillus typhosus were plated and single colonies reisolated. These strains were then subjected to a careful microscopical and cultural examination to determine any subsequent alteration that might result from growth on a medium containing germicides. One transfer from each culture was then made on plain agar, sealed, and placed in the refrigerator. At weekly intervals throughout the course of the experimental work each culture was transferred to drug free agar and, after 24 hours' incubation, was examined microscopically and culturally to determine its purity and any change in morphology or in its cultural reactions which might have accompanied the acquirement of its increased resistance to the germicide. Morphological changes in all thirty drug fast substrains were not infrequently observed but these bizarre forms invariably disappeared after 24 hours' cultivation on drug free agar. No change in the cultural reactions was observed at any time.

The method employed for the attempted production of drug fast strains was as follows: A series of agar slants containing widely varying dilutions of each of the several germicides was inoculated with the five separate cultures, in an

attempt to approximate the maximum concentration of the germicide which would allow the organism to grow. A new series of dilutions was then prepared ranging within narrow limits around that concentration at which growth appeared, in order to establish as nearly as possible the exact maximum concentration of each germicide permitting growth. Three successive daily transfers were made at this concentration to prove that active growth was actually taking place. The concentration thus established was regarded as the original concentration, since any greater strength of germicide prevented continued growth. Each strain was transferred in triplicate throughout the experiments to insure probable growth on at least one of the transfers and to protect against possible contamination. A new series of dilutions slightly greater in concentration were then prepared and the organisms transferred in triplicate from the original concentration

TABLE 1

THE ORIGINAL AND FINAL CONCENTRATIONS, WITH THE PERCENTAGE INCREASE IN CONCENTRATION, OF GERMICIDE PERMITTING ACTIVE GROWTH

Cultures	Germicides					
	Silver nitrate	Mercuro-chrome	Formal-dehyde	Acri-flavin	Hexyl-resorcinol	Phenol
A. B. typhosus (Hopkins)						
Original	200,000	90,000	600,000	20,000	12,000	1,800
Final	90,000	15,000	500,000	1,000	4,000	1,000
Increase, %	55	83.33	16.66	95	66.66	44.44
B. B. coli						
Original	50,000	65,000	150,000	7,000	6,500	900
Final	100	4,000	15,000	600	1,800	600
Increase, %	99.8	93.85	90	91.43	72.31	33.33
C. B. coli						
Original	200,000	65,000	100,000	7,000	4,000	900
Final	100	1,000	20,000	500	2,500	600
Increase, %	99.95	98.46	80	92.86	37.5	33.33
D. B. lactis aerogenes						
Original	150,000	45,000	200,000	7,000	4,500	1,000
Final	100	700	35,000	500	2,500	600
Increase, %	99.91	98.44	82.5	92.86	44.44	40
E. B. coli						
Original	250,000	4,500	100,000	7,000	6,500	900
Final	100	250	20,000	500	1,500	500
Increase, %	99.996	94.44	80	92.86	76.92	44.44

on which they had been growing for five days. If growth appeared on any of these slants of greater concentration, the usual test, consisting of transfers at this dilution for three successive days was applied. If growth continued, a fresh supply of the agar containing the disinfectant at this increased concentration was prepared and transfers again made for a period of five days. When the increased concentration prevented growth the cultures were transferred for another period at the last concentration on which they had grown. The process was repeated until a concentration of each drug was found below which, after repeated trials, each organism failed to grow. This was regarded as the final concentration. After daily transfers for sixty days on this final concentration of each germicide, all attempts to grow the organism on higher concentrations have failed. As five cultures and six drugs were used in this investigation it is evident that a total of sixty sub-strains were developed, thirty growing at the original concentration of the drugs and thirty, which are regarded as drug fast, at the final concentration.

Development of Drug Fast Strains.—An extraordinary degree of tolerance was developed by some of the strains (table 1). Thus, culture E grew originally on a 1 : 250,000 concentration of silver nitrate in agar, but after continued cultivation on this gerimicide it acquired sufficient tolerance to grow on a concentration of 1 : 100. From the data presented in this table it is evident that drug fast strains of bacteria can be developed in vitro.

The length of time required for the development of tolerance for a given germicide by a microorganism, is of considerable interest. The development of drug tolerance did not proceed in the fairly orderly man-

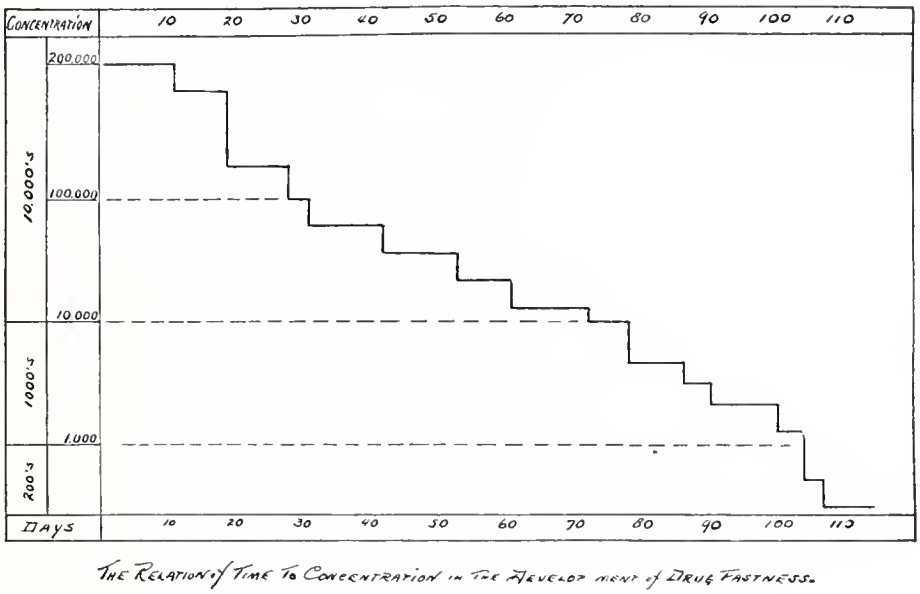
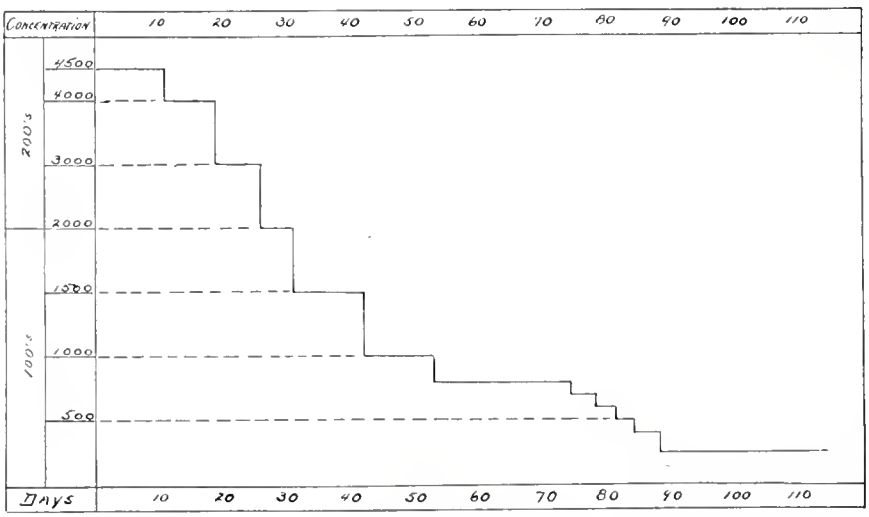


Fig. 1.—B. Lactis Aerogenes and Silver Nitrate.

ner indicated in figures 1 and 2. At times a strain grew on an increased concentration of a drug for three or even five transfers then only to die out. In this case it was necessary to subculture to the next higher dilution on which this strain had previously been growing. After several days cultivation at this dilution it was sometimes possible to obtain continued growth at a considerably greater concentration of the drug than that which had previously prevented growth.

There is marked variation in the degree of drug fastness obtainable as among the six germicides studied. They appear to fall into three fairly sharply defined groups; those to which a high degree of tolerance can be developed, those to which only a relatively low degree of tolerance

can be developed and a group intermediate between the two. Silver nitrate and mercurochrome are in the first group, hexylresorcinol and phenol belong in the second group while formaldehyde and acriflavin fall into the intermediate group. It is of interest to note that the highest degree of drug fastness was developed in those germicides containing a heavy metal, while comparatively little tolerance to germicides of the phenolic type could be developed. This is a point of considerable inter-



The Relation of Time to Concentration in the Development of Drug Fastness.

Fig. 2.—B. Coli (E) and Mercurochrome.

est and is worthy of special consideration owing to the possibility of its clinical application. The original dilutions of silver nitrate and formaldehyde permitting growth were found to be very high but with the exception of Bacillus typhosus, a high degree of tolerance to silver nitrate was developed. The final dilutions of formaldehyde, however, on which the cultures grew remained relatively high. In fact, acriflavin, hexylresorcinol and phenol showed in no case an "original concentration" as dilute as the final or drug fast concentration of formaldehyde. The growth of the Hopkins strain of Bacillus typhosus in the presence of formaldehyde is interesting. This organism proved to be the most sensitive of any to the action of formaldehyde and although repeated efforts were made to increase its tolerance the attempts in this case met with only a very slight degree of success. The concentration of formaldehyde on which Bacillus typhosus would grow was increased from 1 : 600,000 to only 1 : 500,000.

Specificity of the Drug Fast Character.—The fact that bacteria become tolerant or drug fast to various germicides in vitro immediately leads to the question of specificity. If a culture has been developed, drug fast to a specific germicide, is it equally resistant to other germicides? For example, will transfers of culture A drug fast to silver nitrate grow on the final concentration developed for this organism on each of the other five germicides?

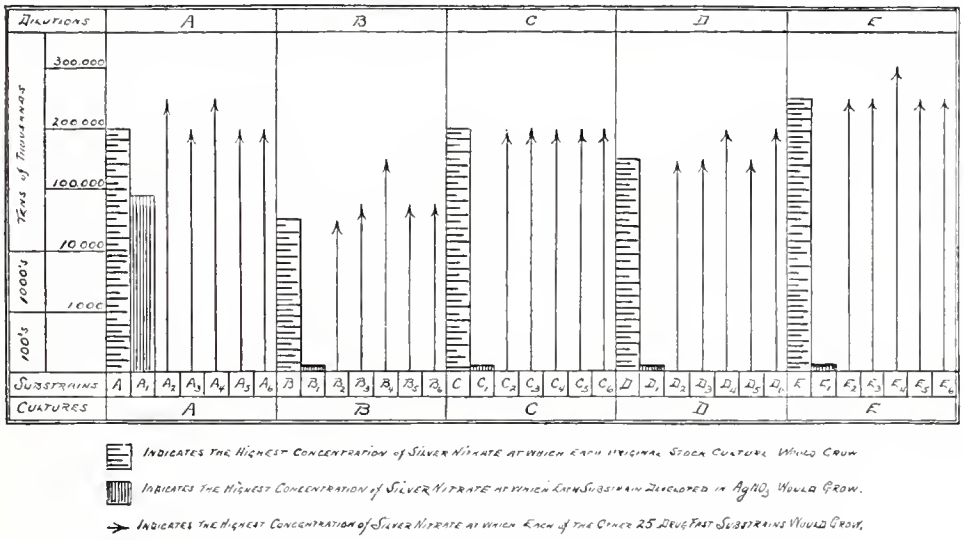


Fig. 3.—B. Typhosus (A), B. Coli (B, C, E), and B. Lactis Aerogenes (D), drug fast to different germicides (1-6),* tested with silver nitrate (1).

The specificity of each of the thirty drug fast strains were tested by the following method:

Subcultures from the final dilution of each germicide were transferred to the final drug fast dilution of that organism on each of the other germicides. Thus, culture A, drug fast to silver nitrate, was transferred to that dilution on mercurochrome, formaldehyde, acriflavin, hexylresorcinol and phenol to which culture A was fast in each case. It is obvious that the drug fast concentration for each of these germicides is different. The usual daily transfers for three successive days were made, in case any growth appeared on the first day, to determine whether active multiplication was taking place.

The drug fast character developed by these organisms is specific. Of the 30 drug fast strains only 5 showed any lack of specificity. Of these, two strains showed a lack of specificity with the drug fast dilutions of three of the germicides and three strains showed lack of specificity with

* See note on page 244.

the drug fast dilution of one germicide (fig. 7 cultures D and E and fig. 8 cultures B, D and E).*

It will be noted that these instances of nonspecificity fall exclusively among the strains drug fast to the two phenolic substances used. As has been already pointed out the degree of tolerance developed with hexylresorcinol and phenol was less than that developed with any of the other germicides. It is possible that in these cases showing an apparent lack of specificity the explanation is to be found in the relatively slight degree of drug fastness developed. It is evident, however, that other factors must enter into any explanation of this lack of specificity in these few exceptions, since in the great majority of these drug fast strains this

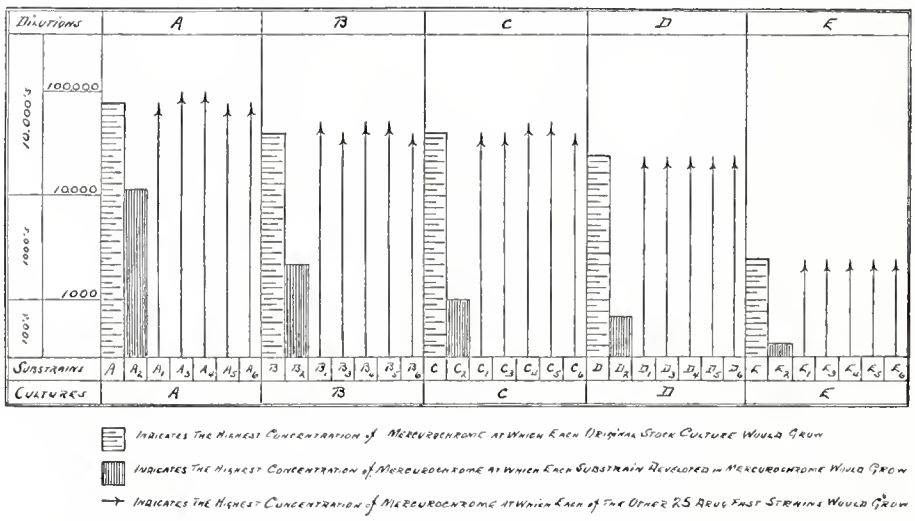


Fig. 4.—All strains drugfast to different germicides, tested with mercurochrome (2).

property is specific in spite of the comparatively low degree of tolerance developed in certain instances.

Resistance of Drug Fast Strains to other Germicides.—The development of a high degree of drug tolerance may indicate some fundamental change in the organism. The remarkable specificity of each strain for the drug to which it has become tolerant indicates to some degree at least the possibility of such a change. Further evidence in support of this view might be offered if organisms fast to one germicide were found to

* The germicides were numbered in the following order: silver nitrate (1), mercurochrome (2), formaldehyde (3) acriflavin (4), hexylresorcinol (5), and phenol (6). The letters referring to the cultures were explained in an early part of the paper. The various combinations of letters and numbers in these tables indicate the specific organism (letter) growing on a particular germicide (number). Thus A1 indicates culture A growing in the presence of silver nitrate, B3 indicates culture B growing in the presence of formaldehyde, etc.

be more sensitive to the action of other germicides. In order to test the relative susceptibility of each drug fast strain to the action of the other germicides, subcultures were made from each drug fast strain, to that concentration of each germicide originally permitting growth.

For example, the six strains of culture A drug fast to each of the germicides were transferred individually to silver nitrate dilution 1 : 200,000 which was the maximum concentration originally permitting growth of culture A. In like manner culture A drug fast to each of the germicides was transferred individually to mercurochrome dilution 1 : 90,000 which was the maximum concentration on which this culture would grow originally. This procedure was repeated for each of the cultures. In short the thirty drug fast strains were transferred individually to the maximum concentration of each germicide originally permitting

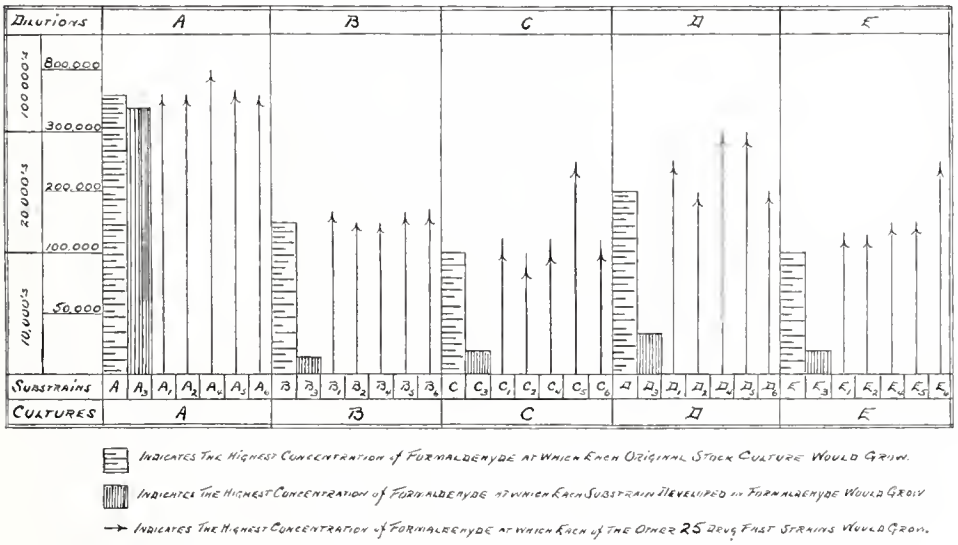


Fig. 5.—All strains drugfast to different germicides, tested with formaldehyde (3).

growth. If no growth was obtained on the original concentration, graded dilutions of decreasing concentrations were made to determine that point at which continued growth could be obtained on three successive daily transfers (figs. 3-8).

In general the drug fast subcultures were found to be more sensitive to the action of formaldehyde, silver nitrate and mercurochrome, than were the original cultures from which they were derived. Sixty seven % of the strains drug fast to the other five germicides were more sensitive to the action of formaldehyde than the original cultures were on first isolation (fig. 5).

For example, culture C grew originally on a dilution of formaldehyde 1 : 90,000 in agar, but after this culture had become drug fast to hexylresorcinol a dilution of formaldehyde to 1 : 250,000 was required before constant growth was obtained.

Approximately 33% of the drug fast strains were found to have developed an increased susceptibility to silver nitrate and mercurochrome (figs. 3 and 4). An average of only 12% of the drug fast strains were found to have become more sensitive to the action of acriflavin, hexylresorcinol and phenol.

In other words, the acquirement by an organism of a markedly increased resistance to the action of a given germicide is not the only evidence, in addition to abnormal morphology and staining reactions, of fundamental changes in that organism. The increased susceptibility of the drug-fast strain to certain other germicides as well as the fact that all of these characteristics can be maintained indefinitely in the proper environment support the view that we are dealing with definitely

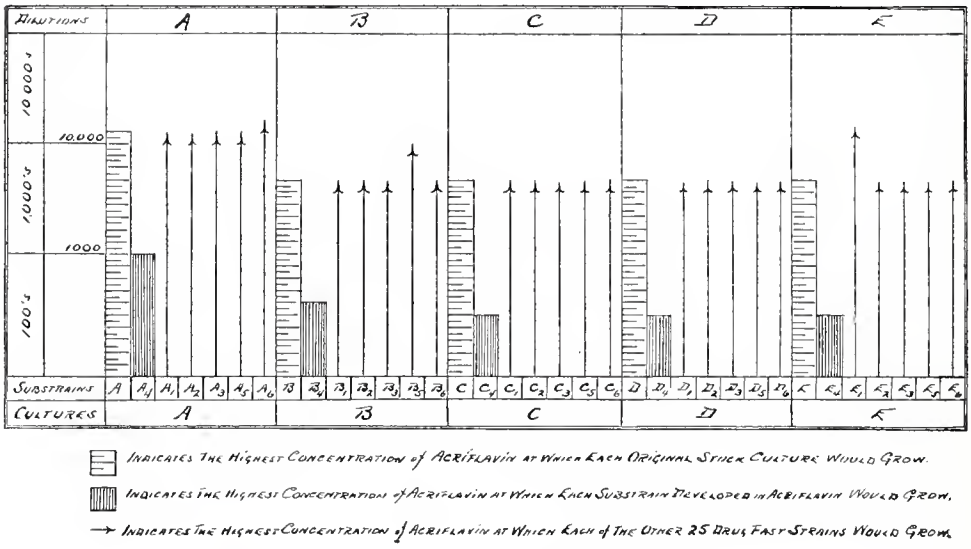


Fig. 6.—All strains tested with acriflavin (4).

acquired characteristics other than individual peculiarities made evident by a process of selection.

In an early part of this paper it was pointed out that a far greater degree of drug fastness could be developed with germicides of the heavy metals than with those of phenolic character and that formaldehyde and acriflavin were intermediate. Of the six germicides silver nitrate, mercurochrome and formaldehyde required the greatest dilutions before continued growth would take place on original isolation. The drug fast strains are even more susceptible to the action of these germicides for in many cases they must be diluted to an even greater extent than for the growth of the original cultures. On the basis of the susceptibility of the drug fast strains to the germicides the same grouping results, for drug

fast cultures are more susceptible to germicides of the heavy metal type than to those of phenolic character. In this case formaldehyde simulates the heavy metals while acriflavin falls in the phenolic group.

Loss of the Drug Fast Character.—It has been shown that certain strains of bacteria can be made highly tolerant or drug fast to certain germicides and that this character is to a large extent specific. The duration of the drug fast character in a drug free environment was next determined. It should be remembered that each drug fast strain was transferred daily to the maximum concentration of the germicide permitting growth and that these strains had been carried in this relatively constant environment for months. Each of these 30 drug fast strains

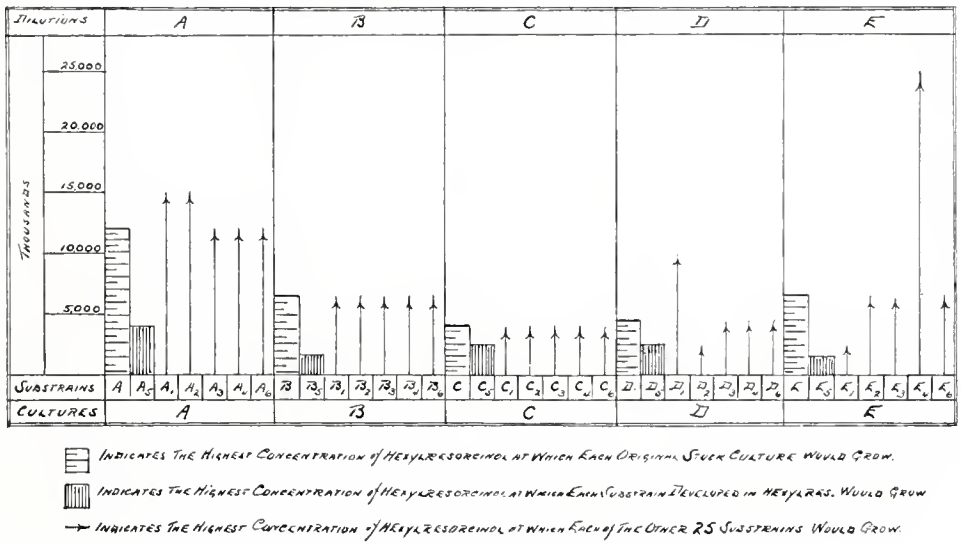


Fig. 7.—All strains tested with hexylresorcinol (5).

was then transferred to drug free agar and subcultured on the same medium daily thereafter. After the first day's growth on plain agar a subculture of each strain was made to an agar slant containing the final concentration of the germicide from which it had been transferred. After the second, third and fourth days growth on plain agar, each culture was again subcultured to its final drug fast concentration as on the first day. Failure to grow on the final concentration of the germicide for three successive days was regarded as evidence of the loss of the drug fast character, although this loss may not have been complete.

Although 80 to 100 days were required to produce the drug fast character in these organisms the capacity to grow on the final maximum

concentration of the various germicides was lost with great rapidity. Eight strains failed to grow after cultivation for only 24 hours on plain agar, four strains after growth for 2 days on plain agar, twelve after 3 days, five after 4 days, and one after 5 days. In other words, 80% of these drug fast substrains failed to grow on the final concentration of germicide after cultivation for three days on drug free agar. Those organisms in which the drug fast character was most highly developed lost their tolerance for the germicide in the shortest time. This interesting point is shown by the fact that only three of all the strains drug fast to silver nitrate and mercurochrome retained their ability to grow on the final concentration of germicide after two days cultivation on drug free

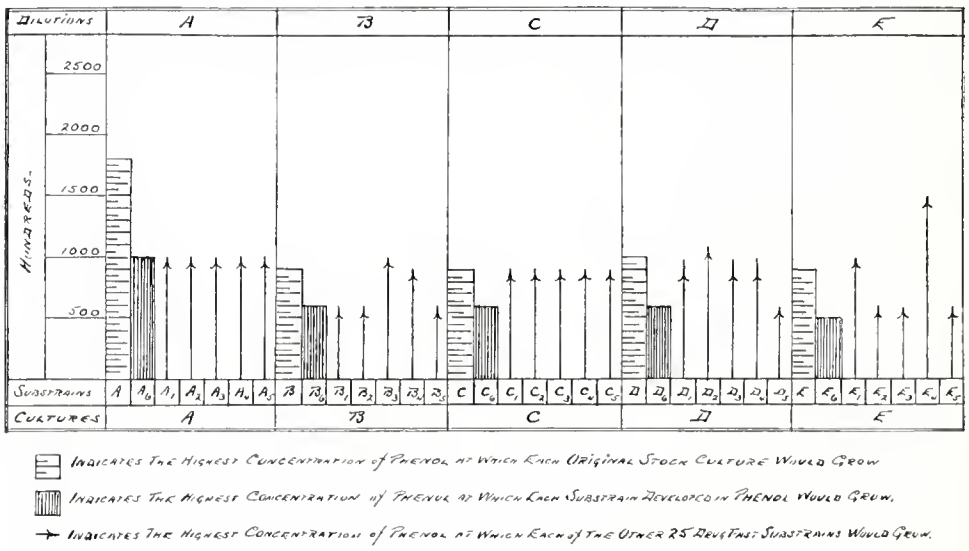


Fig. 8.—All strains tested with phenol (6).

agar and none retained this property after more than three days. All but one of the strains drug fast to hexylresorcinol and phenol continued to exhibit their drug fast character for three days on drug free agar but all lost it after five days.

CONCLUSIONS

Definite drug fastness has been developed in one strain of Bacillus typhosus, three strains of Bacillus coli and one strain of Bacillus lactis aerogenes to silver nitrate, mercurochrome, formaldehyde, acriflavin, hexylresorcinol and phenol.

The highest degree of drug fastness was developed in cultures exposed to the action of germicides containing a heavy metal (silver nitrate and mercurochrome).

When highly developed the drug fast character is specific.

Drug fast strains are, in general, more susceptible to the action of formaldehyde, silver nitrate and mercurochrome than were the original strains on isolation. The majority of drug fast strains do not show increased susceptibility to acriflavin, hexylresorcinol and phenol.

None of the drug fast strains retains its ability to grow on the final maximum concentration of the germicide after cultivation on drug free agar for five days.

All of the organisms of the coli-typhoid group studied in this investigation exhibit wide variations in their behavior toward the various germicides employed.

PHYSIOLOGIC ACTION OF CERTAIN CULTURES OF THE GAS BACILLUS

STUDIES IN BACTERIAL METABOLISM. LXXXI

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The members of the gas bacillus group [1] have been mentioned in association with a considerable variety of conditions, ranging from the rapidly developing, violent gas gangrene, and acute and subacute diarrhea on the one hand, to pernicious anemia,[2] secondary anemia, diabetes, and chronic intestinal intolerance for carbohydrate, on the other hand. The part the gas bacillus plays in most of these conditions is problematical in the light of current information, but it may be said that an unusually abundant growth of the organisms, with extension upwards in the intestinal tract beyond their ordinary habitat is an indication of the absence of normal acidity, or at least, a decrease of normal acidity therein.[3] There is also associated with this overgrowth of gas bacilli a reduction in the number, activity, or both, of the normal lactic-acid producing microbes of the alimentary canal.[4]

The gas bacillus is a versatile organism; it produces a soluble poison, described first by Bull and Pritchett,[5] for which Bull [6] has obtained a specific antitoxin. This poison has been shown to be of a dual nature, as revealed by the work of Henry.[7] One fraction is a hematoxin, inducing laking of red blood cells, which accounts very satisfactorily for the early observation of Herter [8] that the blood of persons who have died from gas bacillus infections shows much hemolysis. The other fraction of the soluble poison is a myotoxin, according to Henry's experiment.

Nor must the observation of Loris-Malekov [9] be overlooked: he inferred from studies of the associated flora of typhoid fever patients that the gas bacillus (called by him B. perfringens, in accordance with

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[1] Simonds: Monograph 5, Rockefeller Inst. for M. Research, 1915.
[2] Herter: J. Biol. Chem., 1906, 2, p. 64. Moench, Kahn and Torrey: J. Infect. Dis., 1925, 37, p. 161.
[3] Kendall: J. Am. M. A., 1926, 86, p. 737.
[4] Kendall, Day and Walker: J. Infect. Dis., 1926, 38, p. 217.
[5] J. Exper. Med., 1917, 26, p. 119.
[6] Ibid., p. 603.
[7] J. Path. & Bact., 1923, 26, p. 497.
[8] J. Biol. Chem., 1906, 2, p. 1.
[9] Ann. d'Inst. Pasteur, 1913, 27, p. 541.

the French nomenclature) caused secondary swelling of the intestinal mucosa and of Peyer's patches. He claims also to have produced typhoid-like swelling of Peyer's patches and mesenteric lymph nodes by intraperitoneal injections of the organisms.

Another substance found consistently in cultures of the gas bacillus in mediums containing utilizable carbohydrate in addition to the usual protein constituents is butyric acid. Much has been written about the possible, or probable, effect of this product of fermentation upon the intestinal movements, and upon the body tissues. McCampbell [10] indeed, attributed much of the reactivity of the gas bacillus to this butyric acid, which the microbe produces in considerable amounts. Some support for this view, in the alimentary canal, at least, seems to be afforded from the experiments of Bokai [11] who injected dilute solutions of various organic acids into loops of the exposed intestine at various levels in anesthetized rabbits. He tried lactic, acetic, succinic, formic, butyric, and propionic acids, and found that each increased peristalsis somewhat. There was some evidence that suitable amounts of relatively low concentrations of these acids induced diarrhea; greater concentrations even incited low grade inflammatory reactions. The discovery of the soluble poison of the gas bacillus, however, has withdrawn attention from butyric acid as a significant reaction product of the organism.

The present communication deals with an entirely different growth product of the gas bacillus—one hitherto not identified, and so far as available literature shows, apparently not definitely detected among its products.

This substance is present in a great majority of strains of gas bacillus which have thus far been studied. It was demonstrated in 66 of 72 strains, obtained from the intestinal contents of 41 persons. It occurs in physiologically demonstrable amounts only in those cultural mediums which contain carbohydrate utilizable for energy, and it has not been detected by this method in corresponding mediums from which carbohydrates have been excluded. The addition of suitable carbohydrates to the latter mediums, however, leads to its appearance within 72 hours, or less.

The physiological effects that have thus far been studied are of two kinds: first, an immediate, considerable contracture in isolated pieces of guinea-pig small intestine suspended in Tyrode solution, according

[10] J. Infect. Dis., 1909, 6, p. 537.
[11] Arch. f. exper. Path. u. Pharm., 1888, 24, p. 153.

to the Trendelenberg [12] technique; and second, a rather abrupt, transitory fall in blood pressure in a dog under ether anesthesia, upon venous injection.

Five hundredths to four tenths of a cc. of a 24 to 48 hour milk culture, filtered through a Berkefeld filter to remove bacteria, will induce the typical, and usually maximal contracture in the isolated gut, where the volume of solution in which it is suspended is not less than 125 cc. Injected intravenously into an anesthetized dog, as little as 0.08 cc. per kilo has produced a drop of 40% in the blood pressure.

If the cultures are incubated for longer periods—up to 7 days—the potency increases somewhat.

The contracture of the isolated guinea-pig gut is a very simple and direct method for detecting this substance, both because it is readily observed and because it is easily recorded. It is the method used for the most part in these experiments.

EXPERIMENTAL WORK

Isolation and identification of cultures.—The bacteria were isolated from suitably diluted suspensions of feces, or duodenal contents, by dilution into milk. The milk was previously heated to expel oxygen, cooled, and heated again to 80 C. for 20 minutes after the addition of the inoculum. Typical, stormy fermentation usually occurred within 18 hours if the organisms were present. The purification of the cultures was attained by plating a small loopful of the milk culture upon a blood agar plate. The process was twice repeated, subculturing between plate cultures in milk. All of the strains proved to be quite hemolytic. This is not wholly in accord with the experience of others,[13] but the results seem unequivocal, although they do not, of course, preclude the possibility that non-hemolytic strains may yet be encountered. Furthermore, the strains reported here have retained their hemolytic powers, with no discernible diminution, for months, as judged by the size of the clear zone around isolated colonies.

The 72 strains studied here agree in essential details with the Simonds types,[1] both culturally and morphologically. They were cultivated from 41 persons, including cases of pernicious and secondary anemias, diabetes, and a considerable number of those persons exhibiting greater or lesser intestinal intolerance for carbohydrate.[3] No relationship was detected between the type of organism and the condition of the patient.

[12] Arch. f. exper. Path. u. Pharm., 1917, 81, p. 53.
[13] For literature, see Simonds,[1] p. 51.

Cultures were made in plain tryptic digest broth containing some undissolved meat, and digest broth enriched with 2% of glucose, lactose, saccharose, mannitol, starch or glycerol. Milk cultures were also made. The anaerobic flasks described previously,[14] holding 100 cc. of culture medium, were found very satisfactory for the purpose, requiring no supplemental technique to prevent the access of oxygen.

At the proper time—after 24 to 72 hours' incubation—the cultures were filtered, first through filter paper to remove the grosser particles, then through Berkefeld filters to eliminate finer particles and bacteria. The filtrates were stored in Erlenmeyer flasks, stoppered with cotton, and kept cool and dark in the ice box.

It was soon discovered that the acidic constituents, principally butyric acid and carbon dioxide, could be removed from these filtrates by simple distillation without materially reducing the potency of the reactive substance. The volume was thereby decreased to about 30% of the original amount. This reduction in volume was found to increase the apparent reactivity of the substance nearly in proportion to the degree of concentration. This indicates quite clearly that neither butyric acid [10] nor the soluble poison [6] of the organism is concerned as such in this connection. Indeed, the soluble poison described by Bull and by Henry is destroyed by this procedure. Although the distillate contained all the butyric acid, it was quite without action upon the isolated gut.

The pathogenicity of the cultures was not tested in animals. Rabbits tolerated 2 to 3 cc. of the filtered fermentation cultures, however, without obvious distress. The injections were made intraperitoneally.

Physiological activity of the cultures.—The depressor effect of injecting 2 cc. of a 24 hour milk culture, prepared as described above, is shown in figure 1. The dog weighed 24 kilograms and was under ether anesthesia. The injection was made intravenously. The extent and duration of the fall in blood pressure is reminiscent of the effects observed by Abel and Nagayama [15] and Abel and Rouiller [16] with their histamine-like depressor substance obtained from extracts of the posterior lobe of the pituitary gland.

Figure 2 shows the effect of the same amount of filtrate upon the blood pressure after atropin.

It should be stated that the substance in question differs, however, from that described by Abel and his associates in that it cannot be

[14] Kendall, Cook and Ryan: J. Infect. Dis., 1921, 29, p. 229.
[15] J. Pharmacol. and Exper. Therap., 1920, 15, p. 356.
[16] Ibid., 1923, 20, p. 72.

extracted from syrupy residues treated with anhydrous sodium carbonate, dried thoroughly, ground and extracted with boiling chloroform. Indeed, practically all of the potency remains behind in the residue from the hot chloroform extraction. It will be recalled that certain histamine compounds, notably the phosphotungstate, are insoluble in this solvent.[17]

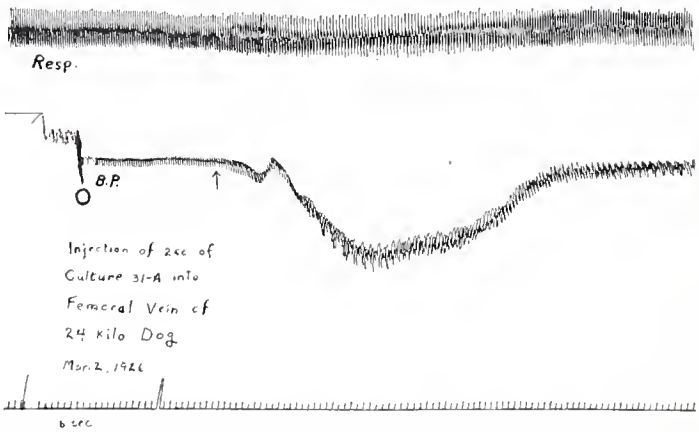

Fig. 1.—Blood pressure and respiration tracings after intravenous injection of 2 cc. of milk culture filtrate.

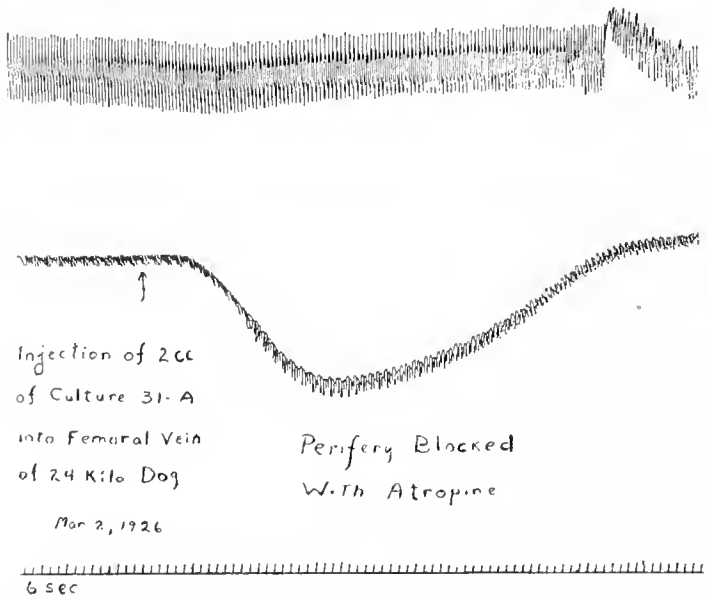

Fig. 2.—Blood pressure and respiration tracings after atropin and 2 cc. of filtrate of milk culture.

It should be emphasized that the depressor effects shown in figures 1 and 2 were obtained with but 0.08 cc. of the crude, unrefined, and unconcentrated filtrate of a milk culture, per kilo dog. Much of this

[17] Drummond: Biochem. J., 1918, 12, p. 5.

volume of fluid is, of course, substance other than the one inducing the reaction. The actual weight of reactive substance must be very little indeed.

Effect on the virgin uterus of the guinea-pig.—Figure 3 shows the contractures induced by the introduction of 0.4 cc. filtrate of a milk culture into 125 cc. Tyrode solution in which is suspended a piece of guinea-pig uterus, arranged to record its contracture.[12] For comparison the contracture induced by 0.05 cc. of a 1 : 1,000,000 solution of his-

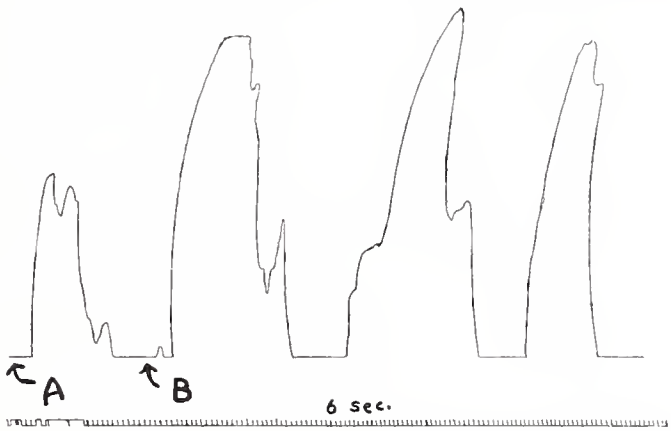

Fig. 3.—Effect on guinea-pig uterus, of 0.4 cc. filtrate of milk culture of gas bacillus in 125 cc. Tyrode's solution.

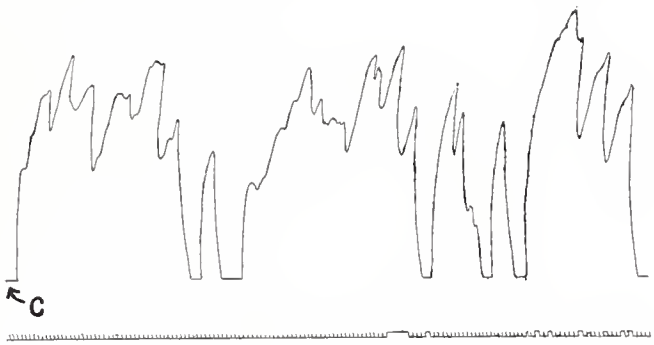

Fig. 4.—Effect on same guinea-pig uterus of 0.05 cc. of 1 : 1,000,000 histamine solution.

tamine hydrochloride in the same piece of uterus is shown (fig. 4). The contracture is qualitatively and quantitatively about the same in both instances, but, of course, this does not in itself prove that the reactive substance in the milk culture is histamine. Between the two tests, the gas bacillus effects were removed by washing the uterus with two changes of Tyrode solution, the time lapse being about twenty minutes. It will be seen that the effects of the first solution—as indicated by the contracture, at least—are removed thus by washing.

Effect on the isolated intestine of the guinea-pig.—Figure 5 shows the prolonged, nearly maximal contracture induced in a piece of isolated small intestine of a guinea-pig when but 0.05 of a 7 day culture of the gas bacillus was added to the 125 cc. of Tyrode solution in which this piece of gut was suspended. Figure 6 shows the contracture curve obtained with another piece of gut: this time, however, 0.2 cc. of the

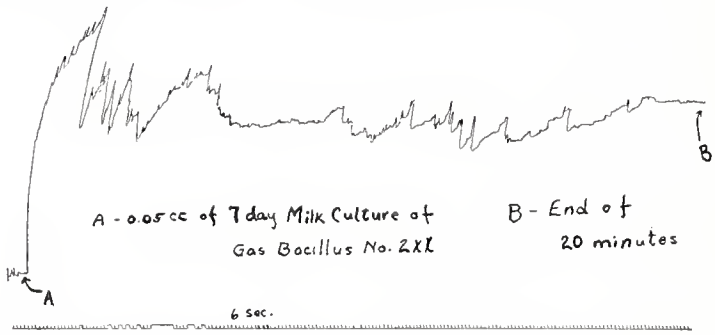

Fig. 5.—Effect on guinea-pig gut of 0.05 cc. of 7 day culture of gas bacillus, in 125 cc. of Tyrode's solution.

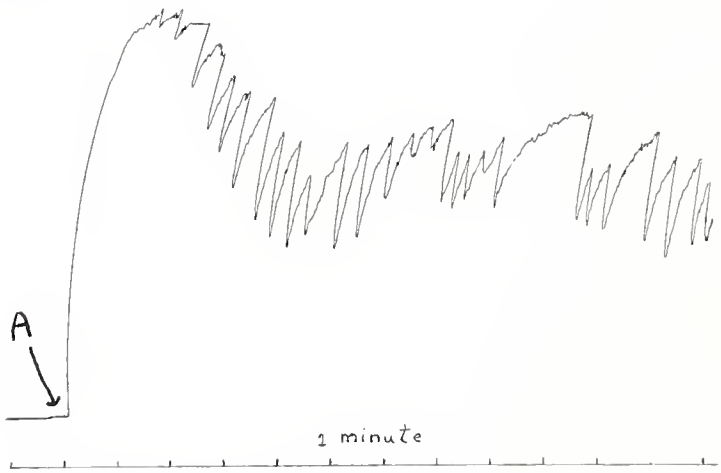

Fig. 6.—Effect on guinea-pig gut of 0.2 cc. of 7 day filtrate.

7 day filtrate was added. After 15 minutes the gut was washed out with fresh Tyrode solution, after which the muscle relaxed. Five hundredths of a 1 : 1,000,000 solution of histamine hydrochloride was then added to obtain a comparative contraction.

Figure 7 is a record of the contracture recorded by this histamine solution. Here again, the similarities of the two curves are quite clearly shown, suggesting pharmacological relationship between the two substances. Cushny [18] has published records of contractures induced by

[18] Heffer: Handb. d. exper. Pharmacol., II, 2, pp. 1325, 1326.

histamine both upon the isolated gut and the uterus of the guinea-pig, which are quite similar to the ones reported here. Nevertheless, in spite of these several apparent similarities, the identity of the reactive substance found in cultures of the gas bacillus with histamine is by no means proved.

A further point of similarity in action between the substance produced by the gas bacillus and histamine salts is afforded by the respective changes they induce in the melanophores of the minnow. Spaeth [19] has suggested that the melanophores of fish and certain amphibia are, in so far as their physiological properties are concerned, much like disguised types of smooth muscle cells. Therefore, it was surmised that the reaction of these cells to the two substances might furnish supplemental evidence of their chemical similarities. Melanophores were gotten from

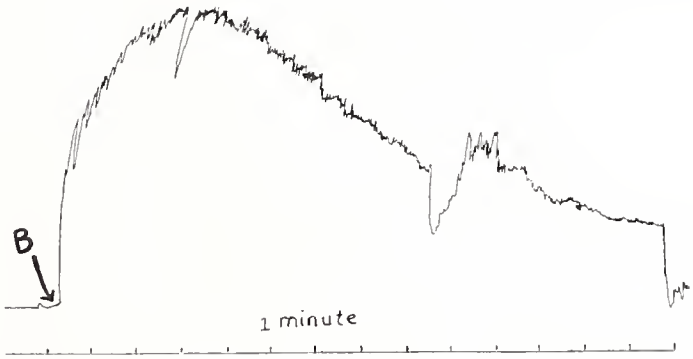

Fig. 7.—Effect on 0.05 cc. of 1: 1,000,000 solution of histamine, on gut washed in Tyrode's solution after treatment with gas bacillus filtrate.

the scales and fins of minnows and placed in a balanced salt solution, according to Spaeth's technique. A small amount of gas bacillus filtrate caused these melanophores to respond with an almost complete contraction in from 5 to 15 minutes. Relaxation could be brought about by transferring these cells to a M/10 NaCl solution. A 1 : 500,000 solution of histamine phosphate also caused the melanophores to contract within a few minutes, thus again indicating its similarity in pharmacological action to the gas bacillus filtrate.

The conditions under which the substance is formed.—Mention has already been made of the fact that the reactive substance, possibly substances, under discussion is detectable only in mediums containing utilizable carbohydrate. Milk, which contains lactose, as well as glucose and saccharose meat broths, and starch or mannitol broth, all contain it,

[19] J. Exper. Zool., 1916, 20, p. 193.

provided the particular strain of the gas bacillus ferments these substances, and it occurs in considerable amounts, or more accurately, in considerable potency, even after 18 hours' incubation. Its amount apparently increases somewhat with length of incubation up to at least 7 days.

This substance is quite resistant to heat, and it is not butyric acid. The latter may be removed quantitatively by distillation leaving a concentrated solution behind which has increased in potency directly with the concentration. The butyric acid distillate is wholly unreactive. Indeed, three to four times the maximal amount of pure butyric, formic or lactic acid that are found in the most vigorous cultures are wholly inert toward isolated gut.

DISCUSSION

The substance itself has many points of chemical resemblance to the aromatic amines: phenylethylamine, indolethylamine, beta-imidazolethylamine; its properties cannot be reconciled with the properties of other known compounds, unless perchance these aromatic amines are linked with such to form di- or poly-peptids.

The mechanism involved in the formation of the reactive substance is apparently of a dual nature. The organism first produces carboxylase from the utilization of the carbohydrates, and this carboxylase in turn decarboxylizes the amino acid, splitting off CO_2, leaving the primary amine behind. This reaction takes place in an acid medium, which according to many observers, is unfavorable for decarboxylation. Hanke and Koessler,[20] however, have shown that proteinogenous amines may be thus formed.

It is rather clear that the enzyme is carboxylase rather than formiase, because the addition of nitrates to the medium prevents the formation of the reactive substance, at least for two or three days. Nitrates inhibit the action of formiase.[21] Formiase and carboxylase are not identical enzymes.

If subsequent study confirms the carboxylase mechanism, the beneficial effects of reducing carbohydrates, feeding butter milk, and increasing protein in the diet of those persons who have symptoms indicative of intestinal intolerance for carbohydrates,[3] will have strong supportive evidence from the chemical and pharmacological view points.

[20] J. Biol. Chem., 1919, 39, p. 539; 1924, 59, pp. 855, 867.
[21] Pakes and Jollyman: J. Chem. Soc., 1901, 79, p. 450.

It will be recalled that these cases have an overgrowth and extension upwards of the gas bacillus. Restricting carbohydrate in their diet, and feeding buttermilk restrains respectively carboxylase production and development of the organisms. Both of these factors should theoretically be helpful in securing clinical relief.

SUMMARY

A histamine-like substance has been described, occurring in a majority of the 72 strains of the gas bacillus thus far studied.

This substance induces histamine-like contractures both in the isolated uterus and the isolated small intestine of the guinea-pig.

A theory of the chemical formation of this substance is proffered.

The theoretical relationship of the substance to certain clinical cases is discussed.

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THE BIOLOGY OF OÏDIUM ALBICANS

WITH SPECIAL REFERENCE TO MYCELIAL PRODUCTION

ONE PLATE

A. A. Draper

From the Department of Bacteriology and Hygiene, Medical College, University of Cincinnati

GENERAL CONSIDERATIONS OF THE BIOLOGY AND CLASSIFICATION OF OÏDIA

Oïdium (Monilia, Endomyces, Saccharomyces) albicans is generally accepted as the specific cause of thrush or parasite stomatitis, a disease of wide distribution. The organism has been found associated, however, with a variety of conditions: general infection involving the skin, nails and lungs,[1] as a normal saprophyte of the stomach [2] in the gastric contents in cases of duodenal ulcer,[3] in laryngeal involvement,[4] in the intestinal tract,[5] in infections of skin and chronic paronychia,[6] and in intradigital saccharomycosis.[7] In the lesions of thrush the organism is found most frequently in the forms of hyphae or mycelia. The elongated cells or filaments range from 30 to 40 microns in length and from 2 to 5 microns in width. They are subject to considerable variation in size and shape. Reproduction occurs by the addition of new hyphae (apical growth) or by division of a single mycelium. In both cases the mycelia are separated by distinct septa in which respect the organism closely resembles the higher fungi. Reproduction may also occur by budding at the septa. The paper deals with the methods used in an attempt to control and fix mycelial and yeast form propogation. The yeast forms are as widely distributed as are the mycelial types. They are consistently found in cultures made on the common laboratory mediums from the lesions of the disease. The yeasts vary little in size. They are globular or ovoid in shape and range from 2 to 3 microns in diameter. They

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[1] Christensen: Am. J. Dis. Child., 1923, 26, p. 450.
[2] Moppart and Kagan: Rev. med. Suisse Romandie, 1923, 42, p. 505.
[3] Merke: Beitr. z. klin. Chir., 1924, 130, p. 549.
[4] Maciucescou-Manoel: Arch. de med. enf., 1925, 28, p. 231.
[5] LeDantec: Compt. rend. Soc. de biol., 1908, 64, p. 106. Bahr: Brit. M. J., 1914, 2, p. 171.
[6] Shelmire: Arch. Dermat. & Syph., 1925, 12, p. 779.
[7] Zwick: Personal communication.

usually reproduce by budding but at times by hypha and division. Ascs have not been observed. Both types of cells stain readily with ordinary aniline dyes. Archibald's stain is very efficient. On the common laboratory mediums (optimum reaction P_H 7-6.5) the organism grows in large white glistening colonies, with slightly convex surfaces and regular edges (fig. 4). Under these conditions reproduction invariably occurs by yeast formation, special methods being required to grow the mycelial forms.

Recent researches have brought to the light the probability of the existence of multiple species of the "thrush fungus." [8] Castellani has shown by means of fermentative reactions that several strains of Monilia albicans differ in their ability to ferment sugars as shown in table 1.

TABLE 1

Fermentations of Sugars by Seven Strains of Monilia (after Castellani)

Monilia Strains	Milk	Glucose	Levulose	Maltose	Galactose	Saccharose	Lactose	Mannite	Dextrin	Raffinose	Arabinose	Gelatin	Glycerine
1	0	AG	AGv.s.	AGs.	As	0	0	0	0	0	0	0	0
2	C	AG	AG	AGs.	A	A	0	0	As.	0	0	0	A
3	C	AG	AG	AGs.	AG	A	A	A	A	0	0	0	0
4	C	AG	AG	ALK	A	AG	A	0	0	0	0	0	0
5	C	AG	AG	A	A	A	A	0	0	0	0	0	A
6	C	A	AG	AG	A	0	0	0	0	0	0	0	0
7	C	AG	0	AGv.s.	A	A	A	0	A	A	A	0	A

TABLE 2

Fermentation of Sugars by Yeasts of Oïdium Albicans

Oïdium albicans Strains	Arabinose	d-Xylose	Glucose	Galactose	Levulose	Lactose	Raffinose	Dextrine	Inulin	Mannite	Gelatin	Milk
1.....	0	0	AG	A	AG	0	0	0	0	A	0	0
2.....	0	0	AG	A	AG	0	0	0	0	0	0	0
3.....	0	0	AG	A	0	0	0	A	0	0	0	0
4.....	0	0	AG	A	AG	0	0	0	0	A	0	C
5.....	0	0	AG	A	AG	0	0	0	0	0	0	0
6.....	0	0	AG	A	0	0	0	0	0	0	0	0
7.....	0	0	AG	A	AG	0	0	0	0	A	0	C

He states that seven different species of fungi belonging to the genus Monilia were isolated from eleven cases of thrush in London. My experiments on the fermentation of sugars (table 2) disclosed similar variations in the zymogenic powers of seven strains of Oïdium albicans isolated at the Cincinnati General Hospital. Such differences may be due either to the loss or gain of fermentation powers following repeated subculturing or, as Castellani believes, to a plurality of species.

Classification.—The true relation of Oïdium albicans to Saccharomyces is as unsettled as is the relation of the yeasts to the fungi.

[8] Castellani: Ann. de l'Inst. Pasteur, 1916, 30, p. 149.

In the vegetable kingdom, the fungi are embraced by the two phyla, Schizomycetes and Thallophyta. In Thallophyta are included the algae, the division fungi and the lichens. The division fungi are grouped into three classes, Phycomycetes, Ascomycetes, and Hyphomycetes or "Fungi Imperfecti." The relationship of Ascomycetes and Hyphomycetes to each other has been for some years the center of attack of conflicting opinions. The morphologic variations of the organisms in these classes and the presence or absence of the ability to produce ascs have been the sole means of differentiation. Ascomycetes includes the organisms that reproduce by means of ascs and ascospores. In this class belong Saccharomycetes or the "true yeasts." Hyphomycetes includes all the species which, chiefly, either because of morphology or the failure of ascs to be observed, cannot be included under Ascomycetes. Saccharomycetes (family) includes those genera represented by unicellular fungi, which multiply by budding, at times by partition, and regularly by the production of ascs. The ascs are spore cases that usually contain from four to eight ascropores. The ascropores have the ability to germinate to a vegetative cell. These organisms are the so-called true yeasts and can be further classified by biochemical and physiological differences. Hyphomycetes on the other hand includes all those genera which have not been seen to produce ascs. The organisms of this group are frequently found as mycelia. The species of this class are not so easily differentiated by biochemical or physiological methods. The classification depends mostly on morphologic differences which in many respects may closely resemble those of the true yeasts or higher fungi.

The place of Oïdia among the fungi is questionable. Because of a failure to produce ascs it cannot be classified under the family Saccharomycetes and takes its place by general agreement under Hyphomycetes. The genus has been subjected to various names and descriptions. The differences in opinion have been based upon the dual yeast-mycelial rôle of propagation. Oïdium albicans was discovered by Robin.[9] The ability of Oïdium albicans to change at the slightest provocation from the mycelial type of cells as isolated from thrush lesions to yeasts has long been regarded as an interesting phenomenon. The opinions of the earlier workers concerning this change have already been set forth.[9] The question of classification of this species revolves around the work of Guilliermond.[10] Do yeasts make up an autonomous species or do they simply represent a stage in the development of the filamentous fungi which are more advanced and may exist at times as yeasts and at other times as mycelia?

STUDIES ON MYCELIAL PRODUCTION

A strain of Oïdium albicans, OA_1, was isolated from the stool of a patient with chronic diarrhea. A former paper discusses the methods of cultivation and the production of mycelia in an aqueous extract

[9] Draper: J. Infect. Dis., 1924, 34. p. 631.
[10] Tanner: Yeasts, 1920, p. 131.

of carrots.[9] Further investigation led to the conclusion that something in the ash of carrots supported the production of mycelia of Oïdium albicans.[11] Specimens of carrot ash were therefore analyzed by Frank S. Andress (1) and R. A. Brown (2) of the University of Cincinnati, and the results given in table 3.

An unexpectedly high proportion of phosphorus was found to be present. It is significant in view of the fact that experiments reported later in this paper had demonstrated the best mycelial growth when phosphorus was present.

TABLE 3

ANALYSES OF CARROT ASH

	Chemical Compound	%
1.	Silica	1.22
	Sulphur trioxide	4.20
	Carbon dioxide	15.80
	Phosphorus pentoxide	12.90
	Calcium oxide	5.17
2.	Carbon dioxide	16.5
	Silica	1.45
	Iron	0.08
	Aluminium	1.00
	Phosphorus pentoxide	8.22
	Calcium	6.40
	Manganese	0.40
	Magnesium	2.75
	Sulphur trioxide	3.80
	Chlorine	2.64
	Sodium	0.79
	Potassium	51.37
	Oxygen (by difference)	4.65
		100.00

Adaptation to Peptone Solution.—OA_2 a second strain of Oïdium albicans was isolated from a case of thrush in February, 1925. In all previous experiments 1% peptone solution had been used as a control medium. Under ordinary circumstances mycelial cells did not develop in this medium.

As a further check on the value of peptone as a control medium the two strains, OA_1 and OA_2 were grown in peptone solution for 35 days. Transplants were made daily or as soon as abundant growth appeared. OA_1 after 32 days developed mycelia and further subcultivation increased the abundance of mycelial production. OA_2 at no time developed mycelia. Both organisms were grown under the same conditions in Witte's and in stock peptone (Parke-Davis). OA_1 developed hyphae in Witte's peptone after 30 days, but OA_2 did not.

[11] Draper: J. Infect. Dis., 1925, 36, p. 484.

Influence of Various Metals and Their Phosphate Salts.—Since the factor that influenced the production of mycelia in carrot infusion appeared to be in the ash of carrots, solutions of the following salts, KSCN, $Fe_2(SO_4)_3$, $AgNO_3$, $Si(NO_3)_2$, $CuBr_2$, HgI, Pb acetate, $HgCl_2$, $SnCl_2$, were prepared by weight in peptone solution with peptone solution as a control. Strain OA_2 which did not produce mycelia in peptone solution was used. A slight production of mycelia in a solution of ferric sulphate (1 : 1,000,000) led to an experiment using various soluble salts of iron. Ferric phosphate in dilution of 1 : 10,000 in peptone solution supported remarkable mycelial growth after 4 days at 37.5 C. The other iron salts gave negative results except for the occasional development of rudimentary mycelia.

TABLE 4

INFLUENCE OF PHOSPHATE SALTS ON MYCELIUM PRODUCTION BY STRAINS OF OÏDIUM ALBICANS

Compound	M Solution	M/2 Solution	
	Growth 14 Days After Inoculation with Yeasts, OA_1		
$Ca_3(PO_4)_3$	±	+++	
$CaH_4(PO_4)_2.H_2O$	+	+++	
$Cr_2(PO_4)_2.6H_2O$	±	++	Later experiments with higher dilutions showed mycelial growth
$FePO_4.4H_2O$	±	±	
$Pb_3(PO_4)_2$	±	++	
$Mn_3(PO_4)_2.7H_2O$	++	+++	
K_3PO_4	+	++	
K_2HPO	+	+++	
$Na_3PO_4.12H_2O$	++	++	
$Na_2HPO_4.12H_2O$	++	++	
$NaH_2PO_4.H_2O$	0	++	
$Na_4P_4O_{12}$	±	+	
$Na_2HPO_3.5H_2O$	±	+++	
Carrot infusion control	++	+++	
Peptone control	±	±	

0, no growth; ±, yeasts only; + to +++, scant to abundant mycelia, in all tables.

Phosphate Salts and Mycelial Production.—The preceding experiment hinted at a probable influence of phosphates on mycelial production. Several soluble phosphate salts were selected and M and M/2 solutions prepared in 1% peptone solution. The solutions were inoculated with OA_2 yeasts and cultivated simultaneously. Mycelial growth occurred in all solutions, as shown in table 4. To check the possible effect of the metallic radicals of these salts, equivalent weight solutions of the metallic radicals but with different anions were prepared in 1% peptone, and inoculated with yeasts and mycelia of OA_2. No mycelia developed. Phosphate salts of the same metals used above, and in equivalent phosphate content, were dissolved in 1% peptone and these supported mycelial growth as shown in table 5.

Effect of Phospholipins.—Phospholipins when suspended in appropriate amounts in peptone solution will support mycelial development equally as well as will inorganic phosphates. Table 6 shows the ability of cephalin and lecithin to support mycelial growth in various dilutions of peptone.

TABLE 5

Mycelial Production in Peptone Solutions, with Equivalent Weights of Phosphates, Inoculated with Yeasts

Compound	Grams of Salt in 100 Cc. Peptone Solution	% Phosphate	Growth from Yeasts OA_2 from Cultures on: Carrot, 1 Month, 5 Days	Carrot, 1 Month, 7 Days	Carrot Infusion 24 Hours, 7 Days	Carrot 24 Hours, 7 Days
$CaH_4(PO_4))_2.H_2O$..	4.3 (in 1000 cc.)	3.25	±	±	++	++
	.43	.325	±	±	++	++
	.043	.0325	±	+	++	++
$FePO_4.H_2O$.....	16.9	3.25	±	±	±	±
	1.69	.325	+	++	++	++
	.169	.0325	±	±	+	++
Pb_3PO_4.....	14. (in 100,000,000 cc.)	3.25	±	±	++	++
	1.4	.325	±	±	++	++
$NaH_2PO_4.H_2O$.....	6.5	3.25	±	±	±	±
	.65	.325	++	++	++	++
	.065	.0325	±	+	+	+
Peptone control....	.....	.....	±	±	±	±

TABLE 6

Effect of Phospholipins on Mycelial Growth from Yeasts of OA_2

Phospholipin	Dilutions in 1% Peptone Solution: 1:100	1:10,000	1:100,000	1:1,000,000	1:10,000,000
Cephalin.....	++	± unexplained	+	+	+
Lecithin.....	+++	++	+	+	±
Carrot infusion (control).....	+++	+	+	±	±

Incubated 48 hours at 37.5 C.

Phosphates in Solid Mediums.—Thus far successful mycelial cultivation had been confined to liquid mediums. An attempt to grow mycelia on agar, with the addition of proper amounts of phosphates met with considerable success when special partial tension methods were used. I have shown that mycelia develop in carrot infusion at a given distance below the surface of the medium.[9] The same conditions prevail in the cultivation of the organisms in agar.

Test tubes containing 10 cc. of cooled melted agar, P_H 6.5, to which had been added 2 cc. of M/2 solutions of various phosphate salts, were inoculated with yeasts OA_1 and OA_2. The contents of the tubes were poured into petri

dishes and allowed to solidify. A layer of sterile agar was superimposed on the seeded layer. The plates were incubated at 37.5 C. In twenty-four hours colonies with mycelia were visible with a 16 mm. lens, and rapidly developed till the mycelia were visible to the eye. If the seeded layer was not covered by a layer of agar, the surface colonies developed yeast forms rapidly and apparently this growth interfered with the development of the deeper colonies. If one seeded the plates with mycelial threads, colonies of this type appeared rapidly. Thirteen soluble phosphates were used. Those of Ca, Fe, Pb, Mn and Na were most favorable, while those of K and Cr gave unfavorable growth.

It would appear that phosphates in the presence of a suitable oxygen tension do influence mycelial production, and, as shown under the respiration experiments, CO_2 also seems to play a rôle.

Type Colonies.—Although the hyphae growth in various phosphate agars under partial tension was general, their mode of development differed so much as to yield entirely different types of colonies. For the purpose of studying more in detail the factors influencing this variation, the salts that supported three types of growth, respectively, were selected: iron phosphate, sodium phosphate and carrot infusion.

Carrot infusion gave rise to colonies termed type 1 (fig. 1), which consist of a multitude of yeasts from which proliferate hair-like filaments after five to seven days of incubation. Subcultivation of such colonies was accompanied by a decrease in the time needed to produce mycelia.

Iron phosphate agar gave rise to type 2 colonies (fig. 2), the early appearances of which are similar to those of type 1. After six to eight days, however, small tufts of mycelia resembling tufts of grass shoot out from the sides of the colony. These tufts grow into long single hyphae which, in turn, give rise to lateral branching at an angle of about 30 degrees. The longitudinal hypha along with its branches takes on a frond-like appearance.

Type 3 colony (fig. 3) develops in sodium phosphite agar. It differs in that it is usually slower in development. After ten days' incubation spine-like projections grow into the medium from a central mass of yeast cells. These projections are characterized by a single longitudinal filament but differ from type 2 in the production of yeasts instead of mycelia along the axis. A fifteen-day old colony has much the shape of a starfish. It is usually from one-fourth to one-half the size of the other types. Repeated experiments showed that the types developed consistently in their given mediums.

Respiration.—Aerobic plate cultures of Oïdium albicans in 10 cc. of agar, to which had been added 2 cc. of a M/2 solution of calcium monophosphate, supported the growth of colonies throughout the depth of the medium. On the surface yeast colonies alone developed. At the bottom of the medium and on the inner surface of the dish yeast colonies likewise developed. After 5 days incubation mycelial threads proliferated from the deepest colonies up into the medium. Colonies midway between the surface and the bottom of the medium developed hyphae. This phenomenon suggested the use of partial tension methods.

To determine, if possible, the exact CO_2-O_2 equilibrium necessary for the best mycelial development, cultures were made on the surfaces of various phosphate agar plates in pairs. One series was incubated aerobically. The other series in a dessicating jar containing some carbon dioxide. Little difference was noted in the amount of mycelial growth with the exception that under CO_2 the mycelial development occurred about twenty-four hours earlier than in the aerobic series. Mixtures of CO_2 and air in proportions of 1:5, 2:4, 3:3, 4:2 and 5:1, as roughly determined by volumetric methods, were placed over agar slant cultures containing no added phosphates. In mixtures of one part air to five of CO_2, growth was accelerated. After seven days, mycelial growth appeared in all cultures (table 7).

TABLE 7

MYCELIAL GROWTH OF OA_1 WITH DIFFERENT PROPORTIONS OF CO_2 AND AIR

		Mycelial Development in Days			
Parts CO_2	Parts Air	2	3	5	7
1	5	++	+++	++++	++++
2	4	++	+++	+++	++++
3	3	0	+++	++++	++++
4	2	+++	+++	++++	++++
5	1	++++	++++	++++	++++

No phosphates added. Incubated at 37.5 C.

More carefully prepared mixtures of air and CO_2 were used to displace the air in rubber corked and pariffin sealed test tubes containing meat infusion agar slants, to which had been added no extra phosphates. The slants were inoculated with yeasts. Twenty different dilutions were made. In nineteen parts of CO_2 and one part of air growth was inhibited. In nineteen parts of air and one part of CO_2 mycelial production was abundant on the sixth day, but growth took place slowly. The optimum mixtures seemed to be sixteen parts of air and four parts of CO_2, in which abundant growth and mycelial production occurred at once. The marked change produced in the gas content of the tubes by the respiration of the organisms makes it impossible to analyze the results accurately.

In another experiment nitrogen was mixed with air in the same proportions as in the CO_2 experiment. Here growth and mycelial production occurred early in a mixture of nineteen parts of N_2 and one part of air; but in nineteen parts of air and one part of N_2 no mycelia were produced; all were yeasts. Evidently if reduced O_2 tension and CO_2 influence the production of mycelia they exert this influence chiefly at the beginning of growth, and before the growth of the microorganisms has altered the mediums.

It now appears that mycelial production of Oïdium albicans depends on either of two factors, or both: the presence of soluble non-toxic phosphates and a low oxygen tension, or this combined with a high carbon dioxide tension, and of favorable tensions of O_2—CO_2, or of O_2—N_2. May not the disturbance of this delicate balance be accountable for pleomorphism of Oïdium albicans?

EXPERIMENTS RELATING TO TYPE COLONIES OF OÏDIUM ALBICANS AND AN ATTEMPT TO FIX SUCH COLONIES ON VARIOUS MEDIUMS

The different types of colonies produced by Oïdium albicans have already been discussed. At the time that these types were observed no particular emphasis was placed on them other than that they represented mycelial production of the organism on agar containing phosphates. Pleomorphism is not an unusual observation; many organisms have been noted to produce under certain conditions colonies that were different from the usual in appearance. Several investigators have, by different methods, successfully bred and fixed type colonies. An example of this is the work of Jordan,[12] on the "rough" and "smooth" types of colonies of the paratyphoid bacillus. By selection of a single cell he was able to fix a type. Three types of colonies have been produced with relative consistency by Oïdium albicans on three mediums as follows: In carrot infusion agar the majority of colonies are type 1 (fig 1); in M/2 ferric phosphate agar the majority of colonies are type 2 (fig. 2), and in M/2 sodium phosphite agar the majority of colonies are type 3 (fig. 3). Can a given type be reproduced in 100% purity upon its specific medium by means of selection of type cells? Can one type be fixed either temporarily or permanently by means of chemical or physical agents upon the medium that usually supports another type?

Selection and Passage on Specific Medium.—Yeasts of a 24 hour old carrot culture of Oïdium albicans, OA_1, were seeded in agar mediums containing carrot infusion, iron ($FePo_4$, $4H_2O$) and sodium (Na_2HPO_3. $.5H_2O$), respectively, and incubated under the partial tension conditions previously described, a layer of sterile agar being superimposed on the inoculated agar. In each plate there were developed in 5 to 8 days many colonies that were characteristic in type for their specific mediums. Such type colonies were fished and seeded on slants of medium corresponding to the medium on which they had originally appeared. Subcultures were made on the same mediums daily for a period of 35 days. The organisms, as yeasts, were again seeded into partial tension plates of the same medium and the percentage of true type colonies computed. There appeared to be no marked difference in the percentage before the subcultivation and the percentage recorded after it. As usual, however, type 1, 2 and 3 colonies were always in majority on the carrot infusion, ferric phosphate and sodium phosphite agar plates, respectively.

[12] J. Am. M. A., 1926, 86, p. 177.

Transferring and Culturing Type Colonies "in toto."—Very young type colonies were cut from their respective mediums and introduced into cooled melted agar and shaken to disperse the mycelial cells. The suspensions were diluted and replanted on the mediums as in the preceding experiment. It is obvious that the sole difference in the two experiments was the use of mycelial cells in the latter for seeding the plates. Seventy-five % of the colonies that developed in carrot infusion agar were type 1 and the remaining % included types 2 and 3, and yeast colonies. The growth on the iron medium was unusually rapid and for the first time showed approximately 100% true type 2 breeding. The colonies in the sodium phosphite medium developed slowly, so the percentage of type colonies could not be determined. The experiment was repeated with similar results. It thus appears that in partial tension plates inoculated with mycelia of a type colony the percentage of true type colonies is greater than in those inoculated with yeasts. Is the tendency

TABLE 8

GROWTHS OF HEATED OA_1 ON CARROT INFUSION AGAR

Minutes Heated at 75 C.	Growth After 48 Hours at 37.5 C.
10	Abundant
20	Abundant
30	Good
40	Fair
50	?
60, 70, 80, 90, 100	0
Control, not heated	Abundant

to vary more inherent in the yeast forms of a given strain than in the mycelia of the same strain?

By means of selection, a given strain produces in the iron medium approximately 100% colonies true to type (fig. 5). If, however, this strain is then cultured in carrot infusion type 2 characteristics are lost in favor of type 1. It is thus not possible to fix colony characteristics permanently by continued cultivation upon a single medium.

Fixation of Types by Heat.—The well known experiments of Hansen on the use of heat to fix type variants of yeasts, and those of Pasteur on the anthrax bacillus suggested the following experiments.

Strain OA_1 (yeast type colony, fig. 4) was selected for this experiment. A suspension of the organism was found to be killed at 80 C. in one hour, but withstood 80 C. for thirty minutes. The temperature 75 C. was therefore chosen for the experiment. This necessitated an accurate determination of the time that the organism would withstand such a temperature (table 8).

Forty minutes was thus found to be the maximum time which OA_1 would withstand a temperature of 75 C. In checking the experiment it was noted that

a culture once submitted to 75 C. for forty minutes was more sensitive to heat than originally. Such a culture on reheating to 75 C. was killed in forty minutes, and yet withstood this temperature for thirty-five minutes. A third heating further increased its sensitivity to heat, this strain being killed in thirty-five minutes but not in thirty minutes. A fourth heating of such subcultures reduced the killing time to thirty minutes, growth occurring when heated twenty-five minutes. No higher degree of sensitivity could be reached. This latter strain will be designated S 25-75 to differentiate it from the original S 40-75.

An interesting fact is, that a heated strain (S 25-75) grown in carrot infusion agar and transplanted into the ferric phosphate medium produces type 1 colonies and does not revert to type 2 as did unheated strains. Such results pointed to a permanent fixation of colony characteristics by heat regardless of the medium upon which it was subsequently grown. These results were checked in the following manner:

Two suspensions of OA_1 in carrot infusion were prepared. One was heated to 75 C. for twenty-five minutes, the other was a control, not heated. Both suspensions were plated in carrot infusion agar by the usual partial tension method. Type 1 colonies predominated in both subcultures. The suspensions were also seeded into the ferric phosphate medium in which the usual type 2 colonies developed. In transplants of subcultures of the heated and unheated strains from carrot infusion agar to the ferric phosphate medium type 1 characters were retained in many colonies of the heated strain (fig. 6). When the unheated strain was plated in exactly the same way most of the colonies grew as type 2 colonies as might be expected (fig. 5).

These experiments were repeated three times, and the fixation effects of the heating may be said to last for at least six weeks. It is too soon to say whether the fixation is permanent or not. Thus we apparently fixed type 1 colony in type 2 medium (iron phosphate). Experiments which are incomplete suggest that similar fixation of type 2 colony on type 1 medium (carrot infusion) may be expected. Here, however, difficulties have been encountered because the heated cells of type 2 colonies do not grow out under the partial tension conditions used above. They may be made to appear, however, by inoculating the surface of the seeded plate with yeasts of the same strain, and when they grow they develop as deep type 2 colonies.

SUMMARY AND CONCLUSIONS

Yeasts of Oïdium albicans, when grown in carrot infusion, developed as mycelia below a certain level in the test tube. The ash of carrots contained some substance that influenced such mycelial growth. Analyses of the ash showed that it contained a large amount of phosphorus.

Various phosphate containing salts, in appropriate concentrations in 1% peptone solutions, supported the hyphae growth.

The organisms grew as mycelia in meat infusion agar if a small amount of inorganic phosphate or carrot infusion was added, and special partial tension conditions were maintained.

Various strains of the organism differed in their biochemical and physiological activities. Cells of the same strain when grown in different phosphate mediums developed type colonies that were specific for their respective mediums. Three such types were observed.

A type grown in its specific medium over a considerable period of time did not retain its type characters when transplanted into a medium specific for another type.

Exposure of cells from a given type colony to an appropriate amount of heat tended to fix the type colony characters insofar that they were retained when they were seeded in a medium which usually supported another type. By this method type 1 was fixed on iron phosphate ($FePO_4.4H_2O$) medium, and type 2 was partially fixed on carrot infusion medium. The degree of permanence of this fixation has not yet been determined.

PLATE 1.

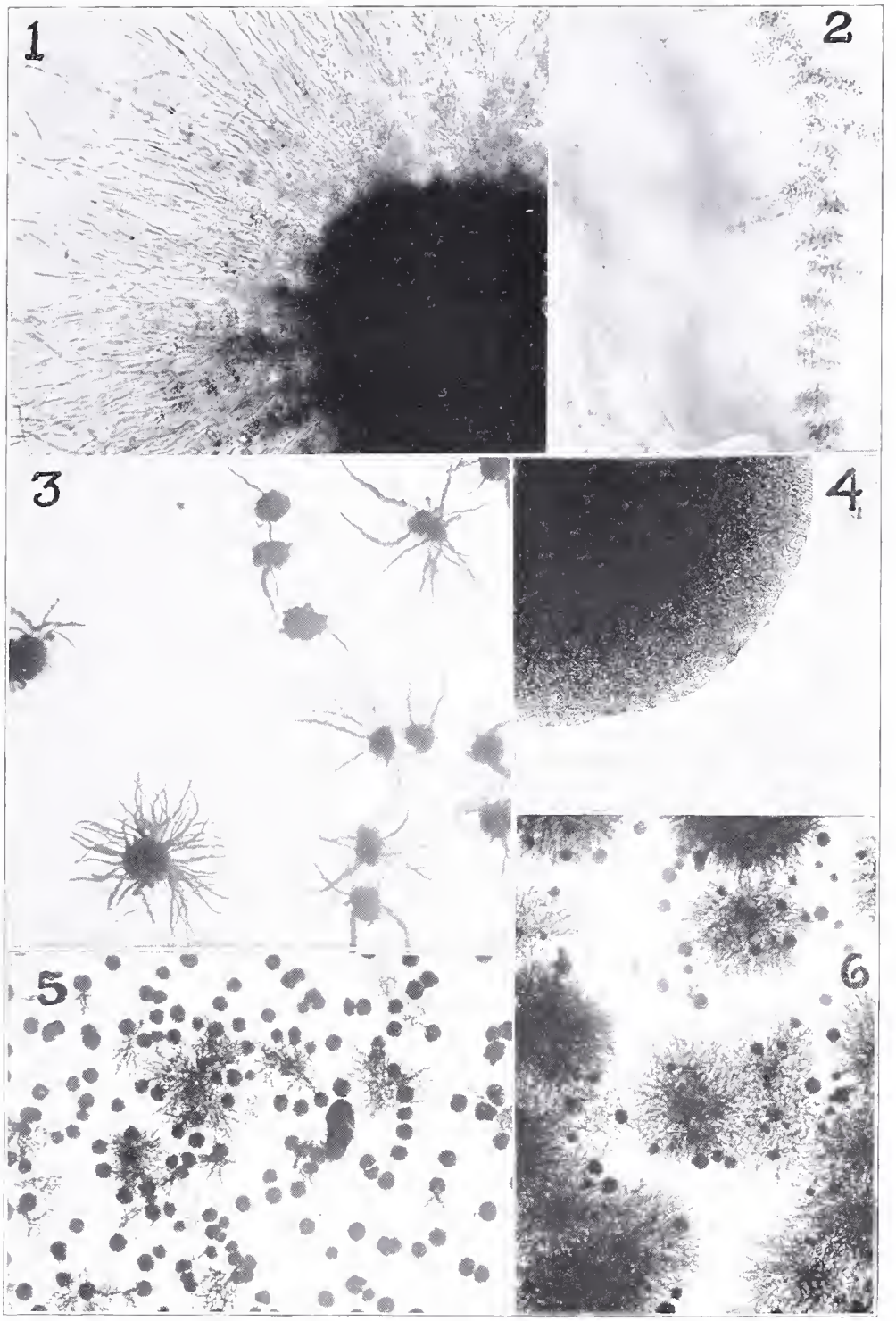

Fig. 1.—Type 1 colony with hair-like mycelia proliferating from a central mass of yeast cells of Oïdium albicans in carrot infusion agar. 8 days old. 75 mm. lens.

Fig. 2.—Type colony with frond-like structure, in ferric phosphate agar. 12 days old. 75 mm. lens.

Fig. 3.—Type 3 colony of star-fish shape, in sodium phosphite agar. 12 days old. 16 mm. lens.

Fig. 4.—Colony of yeasts of Oïdium albicans on meat infusion agar. 48 hours old. 75 mm. lens.

Fig. 5.—Cells of type colonies, subcultured for a series of generations on slants of the type medium and then replated in the same medium, produce nearly 100% type 2 colonies (small). Large colonies are type 1 variants.

Fig. 6.—Cells of type 1 colonies, heated to 75 C. 25 minutes and plated in ferric phosphate agar which favors type 2 colony production, yield a large number of type 1 colonies (large). Small colonies are type 2 variants.

THE INTRAVENOUS USE OF ACRIVIOLET AND OF MERCUROCHROME IN BACTERIAL INFECTIONS

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Since bacteria were first incriminated as the cause of certain infections, the medical profession has sought diligently for some chemotherapeutic substance with which to combat them. Ehrlich's discovery of the spirochetocidal action of salvarsan has especially stimulated an enormous amount of investigation, the object of which has been to find chemicals with a similar specific action in bacterial infections. So far the search cannot be considered successful, and optochin, which kills pneumococci in the blood stream but is too toxic for practical use, is probably the nearest approach to an effective chemotherapeutic agent in septicemia or general infectious conditions.

A number of dyes have been investigated for this purpose and at present certain preparations, including gentian violet, neutral acriflavin and mercurochrome-220 soluble, are of special interest. Their marked antiseptic actions in vitro naturally suggested that they might be of chemotherapeutic value, and has lead to their widespread clinical use. However, a careful study, of the clinical and experimental results reported, impresses one with the fact that while each dye has its enthusiastic advocates, none have proven of great practical value.

In order to obtain further information on this subject an investigation has been made at the Army Medical Center to determine the bactericidal action, toxicity, and chemotherapeutic usefulness of acriviolet, which is a mixture of gentian violet and neutral acriflavin, and of mercurochrome-220 soluble.

ACRIVIOLET

Acriviolet was first studied by Churchman,[1] who found that, in vitro, it had the combined bacteriostatic action of its component dyes—gentian violet and neutral acriflavin. Since the latter substances have been used more extensively than acriviolet as chemotherapeutic agents, a brief review of some of their characteristics is of interest.

Gentian violet, in vitro, inhibits the growth of many gram-positive organisms, includng the pyogenic cocci, but this action is interfered with by protein substances.

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[1] J. Exper. Med., 1923, 38, p. 1.

The dye has been used effectively as a local antiseptic on the skin, in open wounds, infected joints, and in other conditions; but its germicidal value in such infectious processes as empyema or septicemia is doubtful. Churchman[2] and others have reported that gentian violet is comparatively nontoxic for tissue cells; and that rabbits injected with large quantites of the dye were apparently uninjured. Gatch, Trussler and Owen[3] found the dye to be more toxic in aqueous solutions than in physiologic sodium chloride solution. Rabbits injected with the latter solutions survived 5 mg. doses, but a dose of 20 mg. per kilogram of body weight caused death in 9 days. Necropsy showed marked degenerative changes in the kidneys, atrophy of the liver and spleen, fibrosis of the heart muscle and chronic pulmonary congestion. Intravenous doses of 50 mg. per kilogram given to dogs caused almost immediate respiratory failure and death. They also observed that while gentian violet given intravenously had a temporary bacteriostatic effect in the blood of rabbits suffering from staphylococcic septicemia, it did not, in any case, sterilize the blood stream or prevent the death of the animals. Churchman[4] stated that he had never been able to kill organisms circulating in the blood stream by intravascular injections of gentian violet. The reports of the clinical results obtained in treating infections intravenously with gentian violet are contradictory, and even those favorable to the dye are of questionable value because of the lack of suitable control cases.

Acriflavin also has been employed quite generally as a local antiseptic and used intravenously in the treatment of septicemia. Browning and his associates[5] reported that this dye was an effective antiseptic, even in the presence of serum, and that its toxicity was low. Fleming,[6] however, concluded that because of its damaging effect on erythrocytes and leukocytes, the intravenous use of acriflavin, in antiseptic concentrations, was not safe. Spencer[7] found a transitory reduction of erythrocytes and an increase in leukocytes after intravenous injections of the dye, and stated that the bactericidal action of the serum for Staphylococcus aureus was not increased. He was not able to protect mice against pneumococcus infections by intravenous injections of acriflavin. At present a neutral preparation of acriflavin, supposed to be safer than the original substance, is commonly used. Meleney and Zau[8] found that when injected intravenously into rabbits, neutral acriflavin caused leukocytosis, interfered temporarily with the functional activity of the polymorphonuclear leukocytes, had practically no effect on the fragility of erythrocytes and did not change the coagulation rate of the blood. The dye also had a selective action, on tissues of the kidney and liver, which caused it to be taken up by these organs in such concentrations as to injure or destroy them. They concluded that the inhibiting action of neutral acriflavin, even in lethal doses, was so slight, against hemolytic streptococci in the body, that the dye should not be used intravenously in infections due to that organism.

Experimental.—In this investigation of acriviolet the work was taken up as follows: (1) bactericidal action in vitro, (2) local antiseptic action on skin, (3) toxic effect on normal tissues, (4) value in the intravenous treatment of septicemia and general infections in animals and in man. The acriviolet used was manufactured by the National Anilin and Chem-

[2] Ibid., 1913, 18, p. 579.
[3] J. Am. M. A., 1925, 85, p. 894.
[4] Ibid., p. 1849.
[5] Browning, C. H.; Kennaway, E. L.; Gulbransen, R., and Thornton, L. H.: Brit. M. J., 1917, 1, p. 73.
[6] Lancet, 1917, 2, p. 341.
[7] J. Lab. & Clin. Med., 1924, 9, p. 322.
[8] J. Am. M. A., 1925, 84, p. 377.

ical Company. A strain of Staphylococcus aureus, isolated from the blood of a fatal human case of septicemia, was used in all of the experiments.

1. Bactericidal action, in vitro: Acriviolet in vitro is an effective bactericidal substance, in the absence of proteins. Diluted 1 : 1,000 in normal salt solution it killed Staphylococcus aureus in 15 minutes; but in 75% defibrinated blood, a dye concentration of 1 : 200 was required (table 1). To obtain the latter concentration of the dye in the blood stream, even temporarily, would require the intravenous injection of an immediately fatal dose of acriviolet.

Ox bile also interfered with the bactericidal action of acriviolet, so that Eberthella typhi (Rawlings) was not killed by dilutions of 1 : 200 in 2 hours.

TABLE 1

BACTERICIDAL ACTION OF ACRIVIOLET ON STAPHYLOCOCCUS AUREUS (C) AND ON E. TYPHI (R) (CONCENTRATIONS OF DYE REQUIRED TO KILL ORGANISMS AT VARIOUS TIME INTERVALS)

Staphylococcus aureus (C)				
Dye solution in	15 Minutes	1 Hour	5 Hours	20 Hours
Physiologic salt solution	1:1000	1:25,000	1:70,000	1:70,000 or less
75% blood	1:200	1:1000	.....	1:20,000
E. typhi (R)				
Dye Solution in	5 Minutes	30 Minutes	1 Hour	2 Hours
Physiologic salt solution	1:500	1:5000	1:5000	1:10,000
Ox bile	*	*	*	*

* In ox bile, the strongest concentration of acriviolet used (1:200) failed to kill E. typhi, even in 2 hours.

2. Local antiseptic action on skin: From other experiments it was concluded that acriviolet 5% in 50% alcohol was a fairly effective skin antiseptic. Normal unwashed human skin and skin contaminated with dried saliva or throat secretions were sterilized in 2 minutes. However, no spore-bearing organisms were used in these tests. Reddish[9] reported that acriviolet 5% in alcoholic solutions did not kill Cl. welchii on wet filter paper in 2 hours.

3. Toxic effect on normal tissues: (a) Acriviolet injected subcutaneously, or intracutaneously, into rabbits produced locally an intense purplish-black stain, followed in several days by necrosis and sloughing of the skin. A similar necrotic lesion of human skin was observed in one case following the accidental subcutaneous leaking of a 0.5% solution during intravenous injection. After 4 days the skin appeared inflamed with a central area 3x4 cm. which was bluish-black. This dark necrotic area became dry, hard, and wrinkled, and gradually separated, at

[9] Special reports in personal communications, 1925.

its edges, from the surrounding skin. At the end of 6 weeks it was removed and replaced by skin graft.

(b) Aqueous solutions of acriviolet caused hemolysis of human erythrocytes; but salt solutions did not.

(c) According to French, when acriviolet is injected intravenously there appears to be a progressive oxidation of the gentian violet, resulting in the formation of formaldehyde and possibly of formic acid; and only the acriflavin is eliminated through the kidneys without apparent change. Nichols has demonstrated that following intravenous injections of acriviolet in rabbits, gentian violet appeared in the bile in about 10 minutes and was followed later by the acriflavin.

Because of the tendency of the dye to precipitate rather rapidly, especially in salt solution, all solutions were prepared immediately before use, and were protected from light. It was found that 1% solutions of acriviolet in distilled water or in 0.25% NaCl solution were apparently unchanged for over 24 hours, while in 0.85% NaCl solution a moderate precipitate occurred in ½ hour, and a very heavy precipitate in 24 hours. One-half% solutions of acriviolet in normal salt solution were much more stable.

In order to determine the toxicity of acriviolet, various amounts of the dye were injected intravenously into normal rabbits. All of the doses given are recorded in mgs. per kilogram of the animal's body weight. Daily records were made of the appearance and weight of the animals, and in several experiments examinations were made of their blood nonprotein nitrogen and creatinin (Fleming). Gross and microscopic examinations were made of all animals after death, and histologic diagnoses were made by Callender.

Acriviolet, dissolved in distilled water, was found to be more toxic than in normal salt solution. Single intravenous doses of 10, 15 and 20 mg. of the dye, in aqueous solutions, killed rabbits in 15 days, 3 days and 2 hours respectively, while after single doses of 5 and 7.5 mg. the animals survived. Rabbits, after receiving the rapidly fatal 20 mg. doses, were affected almost immediately with severe convulsions followed by great prostration and death. The skin and subcutaneous tissues were stained a pea-green color while the heart, liver, kidneys, stomach, intestines, bladder and conjunctivae were purple. Microscopically, the kidneys showed swelling of the cells of the convoluted tubules, ascending loop of Henle and of the glomeruli. Marked degenerative changes were found in the kidneys of the animal which died 15 days after a single 10 mg. dose.

Acriviolet 0.5%, dissolved in physiologic sodium chloride solution, was given intravenously to rabbits in doses of from 5 to 30 mg. per kilogram. Single 30 mg. doses were followed by death in from 5 minutes to 24 hours; doses of 25 mg. killed 2 out of 3 rabbits in one and three days respectively; and after 20 mg. doses one of three animals died in nine days. While animals were not killed by the smaller amounts, there was evidence that, even in these doses, acriviolet had an injurious effect on the liver and kidneys. Single doses of 5 and 8 mg. were followed by a temporary increase in the blood nonprotein nitrogen and creatinin without kidney lesions demonstrable histologically. However, single doses of 9, 10, 11, 12 and 20 mg. produced not only progressively greater functional disturbance of the kidneys, as indicated by the blood chemistry results, but also increasingly severe degrees of acute parenchymatous degeneration of the liver and kidneys.

The repeated daily injection of 10 mg. per kilo resulted in death after seven doses. The animal lost weight and, as shown in fig. 1, the blood nonprotein nitrogen and creatinin increased progressively to 300 mg., and 7.2 mg., per 100 cc.

respectively just before death. The subcutaneous tissues were stained a pea-green color and the internal organs were purple. Histologically, the liver showed a central necrosis and the kidneys a severe parenchymatous degeneration.

Daily injections of 5 mg. doses were followed by a similar loss in weight, and an increase in blood nonprotein nitrogen and creatinin. When the injections were discontinued after the eighth day, because of thrombosis of the veins, the blood chemistry findings approached normal again, but did not reach their original levels. When this animal was killed one month later the kidneys showed gross and histologic evidences of chronic interstitial nephritis.

The foregoing experiments indicate that if used for intravenous injection, acriviolet should be given in 0.5% strength in normal salt solution; that the dose should not exceed 5 mg. per kilogram and that this dose should not be given on more than two successive days.

4. Value in the treatment of bacterial infections: (a) Rabbits infected with standardized doses of Staphylococcus aureus (C) were treated with various amounts of acriviolet. Untreated controls were observed in each experiment. Records were kept of the appearance and weight of each animal, and, at necropsy, histological and bacteriological examinations were made.

In the first two experiments rabbits, infected by intravenous injections of non-lethal doses of staphylococci, were treated intravenously with aqueous solutions of acriviolet. The untreated control animals lived, and when killed at the end of the month, their lesions were apparently healing. The treated animals received 10 mg. doses of acriviolet on the day after infection and one animal had a second dose on the eighth day. These animals died in from ten minutes to twelve days, with typical staphylococcic lesions in the heart and kidneys, from which massive pure cultures of staphylococci were obtained.

Similar results were obtained in other experiments, in which saline solutions of acriviolet were used for intravenous treatment. Animals infected with lethal doses of staphylococci and treated immediately after infection with 5 mg. doses of acriviolet died in 20 to 48 hours. The untreated control animals in this series died on the third, fourth and fifth days. Typical staphylococcic lesions were found and pure cultures of Staphylococcus aureus obtained from the hearts and kidneys of both control and treated animals. An effort was then made to prolong any possible bacteriostatic action of the dye by administering it at shorter intervals. Infected rabbits were given 5 mg. of acriviolet intravenously immediately after inoculation, followed by similar doses at the end of one and two hours respectively. The animals so treated died in 20 hours, while untreated control animals lived four and five days respectively. Typical lesions were found in all these animals, from which pure cultures of staphylococcus were obtained.

It was concluded that acriviolet injected intravenously did not kill staphylococci or prevent the formation of lesions by them, in rabbits; that its use hastened the death of animals infected with lethal doses of staphylococci, and caused the death of animals infected with non-lethal amounts of staphylococci.

(b) Acriviolet was used intravenously for the treatment of eight cases of bacterial infection, six of which were septicemia. One of

the cases of septicemia was infected with Staphylococcus albus, one with Streptococcus viridans, and four with Streptococcus hemolyticus. The acriviolet solution was prepared by dissolving 0.5% of the dye in hot normal salt solution, filtering and sterilizing it in the autoclave at 15 pounds for 15 minutes. Solutions were kept at body temperature during the injections, which were made very slowly.

Case 1.—L. L. (Capt. J. A. Mendelson): This patient had a rash, accompanied by clinical signs of sepsis, following infection with Staphylococcus albus, of a surgical wound made to repair a ruptured biceps tendon. All blood cultures were sterile. The infected wound was reopened, irrigated and drained; and acriviolet, 5 mg. per kilogram, was given intravenously on 3 successive days. After six days the wound was still draining and contained stapbylococci, the patient's temperature was normal and his general condition much better. On the 19th day the wound had healed and the patient fully recovered. It is probable that the local treatment and drainage of the infecting focus were responsible for the recovery of this patient.

Case 2.—McG. (Maj. B. S. McClintic): Chronic empyema with pleurobronchial fistula (mixed infection), and chronic toxic nephritis. After thoracotomy the patient became critically ill and had a septic temperature. Blood cultures were sterile. He was given, intravenously, 5 mg. of acriviolet per kilogram on the 5th and 6th days, but was not improved clinically. On the latter date two ribs were resected and the patient received a blood transfusion to which he reacted favorably. He continued to be critically ill. The patient was then given mercurochrome-220, 3 mg. per kilogram, intravenously, on the 15th day and died on the 19th.

Case 3.—McR. (Maj. F. D. Francis): Septicemia due to Staphylococcus albus, with acute phlebitis, right hemorrhoidal vein. This patient was ill and had a septic type of temperature on September 16th. Blood cultures were positive October 4th and on October 7th at 2: 00 p. m. Two other blood cultures taken on October 7th at 4: 00 and at 6: 00 p. m. were sterile. On October 9th, 10th and 12th, when patient had almost recovered and was asking to go home, she was given 5 mg. doses of acriviolet intravenously. October 25 she was considered cured and discharged. This patient was practically well when dye therapy was begun.

Case 4.—McGi. (Maj. A. W. Kenner): Septicemia due to Streptococcus hemolyticus complicating a chronic cellulitis of left thigh (Staphylococcus aureus) with many sinuses; and osteomyelitis of left femur (tuberculous). Patient's leg had been treated locally for about 9 months. On October 18th his general condition became worse and the temperature much higher. Blood cultures this date contained hemolytic streptococci, but another taken on October 20th was negative. On the latter date, acriviolet, 5 mg. per kilogram, was given intravenously and the same dose was repeated October 21st, 25th and 27th. This treatment did not cause any improvement of the patient's condition or change in the bacterial flora of the wound.

Case 5.—K. (Maj. W. S. Wells): Septicemia and subacute endocarditis, aortic valve, due to Streptoeoccus viridans. Blood cultures taken December 10th and 12th contained large numbers of S. viridans. December 12th, when temperature was 103.6 F., patient was given intravenously 5 mg. acriviolet per kilogram. His condition became worse on December 13th and he died suddenly on December 15th. Permission for necropsy was refused.

Case 6.—(Maj. J. A. Bethea): Septicemia due to Streptococcus hemolyticus. Clinically this was a case of erysipelas. Blood culture contained hemolytic streptococci, but cultures taken just before treatment were negative. The patient was given 2 doses of acriviolet, 4 mg. per kilogram, 24 hours apart. These treatments caused no improvement, and the patient recovered in about the usual time required in erysipelas.

Case 7.—(Maj. J. A. Bethea): Septicemia due to Streptococcus hemolyticus accompanying cellulitis of the nasal septum following trauma. Hemolytic streptococci were found in blood culture and the patient was given intravenously two doses of mercurochrome-220, 4 mg. per kilogram, 24 hours apart. His condition became progressively worse, and blood cultures contained a larger number of colonies per cc. than before treatment. He was then given two doses of acriviolet, 5 mg. per kilogram, 24 hours apart, following which his condition became much worse, and he died.

Case 8.—(Capt. V. N. Meddis): Septicemia due to Streptococcus hemolyticus. Rupture of the posterior urethra was followed by gangrene of the genitals and perineum. An anerobic gas-forming bacillus was present. The entire gangrenous area was removed surgically. Twenty-four hours later blood cultures contained hemolytic streptococci and the patient was given acriviolet, 5 mg. per kilogram, following which his condition became worse.

Gentian violet was given intravenously, in doses of 4 mg. per kilogram, to two cases of staphylococcus septicemia. One of these, in which the focus of infection had been removed surgically just before the dye was given, recovered. The other patient who received two doses of the dye had more staphylococci in blood cultures than before, became much worse, and died. Major Bethea reported: that during this same period two cases of septicemia, due to hemolytic streptococci, recovered promptly after no special treatment other than surgical removal of the focus of infection. Another, apparently hopeless case of pneumonia, witb septicemia due to Streptococcus viridans, who was considered too ill for intravenous dye therapy, recovered without any specific treatment.

The intravenous use of acriviolet was discontinued because of its apparent lack of therapeutic value, and because of its toxic effect on

the kidneys. Only two cases in this series showed any improvement following treatment; in one of these the only known focus of infection had been treated surgically and drained, while the other case had practically recovered when dye therapy was begun. Acriviolet injections had no beneficial effect on two cases of streptococcus septicemia in both of which the blood cultures were negative before treatment was begun. Four cases, two of which were septicemia due to hemolytic streptococci and one to Streptococcus viridans, became much worse after treatment with acriviolet and three of these patients died.

MERCUROCHROME-220 SOLUBLE

Mercurochrome-220 soluble, the disodium salt of dibromhydroxymercurifluorescein, containing 23 to 26% of mercury, has been used extensively as a germicide in the local and intravenous treatment of various bacterial infections.

Young, Hill and Scott[10] found mercurochrome-220 to be strongly bactericidal in urine, dilutions of about 1 : 1,000, killing B. coli and Staphylococcus aureus in one minute, while in the presence of 50% dog serum, the dye was less active, a dilution of 1 : 100 being required to kill B. coli and 1 : 90 for Staphylococcus aureus. Hill and Scott[11] reported, that, in ox bile, mercurochrome-220, diluted 1 : 5,000, killed E. typhi (Beckwith), diluted 1 : 1,000, killed E. typhi Rawlings, and diluted 1 : 100 killed Staphylococcus aureus in one hour.

Scott and Hill[12] decided that a 2% alcohol-acetone-aqueous solution of mercurochrome-220 was a very effective skin disinfectant, and slightly better than iodine and alcohol. Reddish[9] reported that the dye in 5% alcoholic solutions did not kill Cl. welchii or Cl. tetani in two hours.

Brill and Myers,[13] using culture mediums, enriched with human blood, found that mercurochrome-220, diluted 1 : 10,000, representing the maximum advisable concentration of the dye in the circulation, had no direct bactericidal action in three hours on staphylococci, streptococci or B. coli, in vitro. Walker,[14] working with fresh defibrinated blood, failed to obtain any evidence of a direct bactericidal action, in the usual concentrations of the dye. On the contrary, he demonstrated that mercurochrome-220 interfered markedly with the normal bactericidal and bacteriostatic action of the blood as tested with B. coli, Staphylococcus aureus and Streptococcus hemolyticus. Walker concluded that the use of mercurochrome-220 in septicemia was entirely empirical, and that the dye has no specific action on the causative microorganisms. Moreover, he felt that the interference with the normal bactericidal action of blood constituted at least a theoretical objection to its use. Gatch, Trussler and Owen[3] reported that the intravenous injection of mercurochrome-220 in rabbits, infected with staphylococci, was followed by a temporary bacteriostatic effect; but that it did not sterilize the blood stream. Moreover, they concluded that: "a large dose injected in the presence of an overwhelming infection may hasten death. Todd[15] treated rabbits, inoculated intravenously with B. aertrycke and B. coli, with sublethal doses of mercurochrome-220

[10] Arch. Surg., 1925, 10, p. 813.
[11] Arch. Int. Med., 1925, 35, p. 503.
[12] J. Urol., 1925, 14, p. 135.
[13] J. Am. M. A., 1925, 84, p. 879.
[14] Arch. Path. & Lab. Med., Chicago, 1926, 1, p. 199.
[15] Lancet, 1925, 209, p. 1017.

at various intervals. He concluded that at acidities near to normalcy, the antiseptic properties of mercurochrome are relatively feeble, and that the dye is unlikely to be of value in general infections. All of the treated animals died before the controls did.

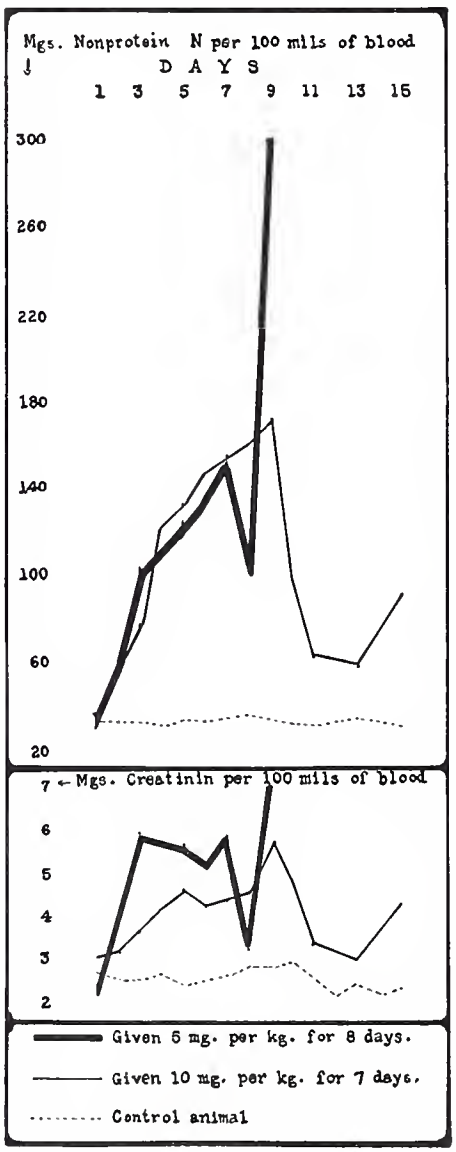

Fig. 1.—The effect of daily intravenous injections of acriviolet on the blood nonprotein nitrogen and creatinin of rabbits.

Young, Hill and Scott,[10] stimulated by the conflicting reports concerning the clinical value of mercurochrome-220, compiled the results of a large number of cases treated with the dye. They concluded that in such diseases of the skin as erysipelas, furunculosis, cellulitis and infections of the soft parts, including abscesses, a very rapid clearing up of the infectious process often followed intra-

venous injections of mercurochrome-220. They also reported the relieving of pain and swelling in arthritis, the recovery of 14 cases of septicemia, and striking results in a limited number of cases of pneumonia in children. In gonorrhea, they decided that the recorded experiences were too meager to warrant making deductions, and advised the additional use of well tried old methods of treatment. In chronic urinary infections, there had been some successes and many failures. They also felt that its use in the treatment of typhoid carriers was warranted.

Dudgeon[16] believed that both bichloride of mercury and mercurochrome-220 were of value in septicemia. However, he considered 10 cc. of a 1 : 200 solution of mercurochrome the maximum dose, and even this should be reduced on repetition. He observed cases of nephritis with blood, albumin, and casts in the urine following injections of mercurochrome-220.

Mercurochrome-220, 0.5% to 1% in aqueous solution, has been used locally on the skin and mucous membranes without producing irritation. After subcutaneous injections, Dewitt[17] observed induration, necrosis and ulceration around the point of injection. Rolnich[18] performed 20 vasectomies on dogs and by injecting 2% and 3% solutions of mercurochrome-220 produced occlusion of the vas, as a result of scar tissue formation, in all but two. Corper[19] showed that intracutaneous injections in dogs, of 0.2 cc. of 0.5, 1 and 2% solutions of the dye, resulted in sloughing and ulceration in about seven days. Intrapleural injections, even of 0.1% solutions, resulted in marked hemorrhagic pleuritis, and acute hemorhagic pneumonia. Walker[14] has shown that the dye is markedly hemolytic when added to washed red blood cells suspended in salt solution, but that the presence of serum prevents this action, except in dilutions of 1 : 100 and 1 : 200. Young, and his associates,[10] reported that mercurochrome-220, injected intravenously, was of about the same toxicity to human beings as to rabbits, and that the latter were invariably killed in 24 hours by doses of 10 mg. per kilogram. Rabbits receiving 5 mg. per kilogram showed a decrease in phenolsulphonphthalein elimination, and albuminuria, for about one week. Human beings were apparently not injured by 5 mg. doses, but larger doses caused reactions characterized by nausea, vomiting and frequent stools; sometimes a slight albuminuria and occasionally a few casts in the urine, which disappeared in a few days. Reed and Lum[20] observed that the intravenous administration of mercurochrome-220 in the treatment of bacterial septicemia, was followed by the usual clinical features of mercurialism. St. George[21] has reported the autopsy findings in five cases of sepsis, treated intravenously with mercurochrome-220, and stated that, in his opinion, the drug induced intense nephritic and intestinal lesions, and thus contributed to death. Larger amounts of mercury were found in the viscera of these cases than is commonly found in cases of mercuric chloride poisoning.

Experimental.—1. Bactericidal action—in vitro: The bactericidal effect of mercurochrome-220, in vitro, on Staphylococcus aureus (C) and on E. typhi (R) was determined under various conditions. Mercurochrome-220, diluted 1 : 100 in water, killed staphylococci in 15 minutes, and diluted 1 : 20,000, it killed in 1 hour (table 2). However, when blood was added, a 1 : 100 dilution of the dye did not kill the organisms

[16] Ibid., 1926, 210, p. 169.
[17] J. Am. M. A., 1920, 75, p. 1422.
[18] J. Urol., 1924, 12, p. 445.
[19] J. Am. M. A., 1925, 85, p. 256.
[20] J. Am. M. A., 1924, 83, p. 1681.
[21] Ibid., 1925, 85, p. 2005.

in 1 hour. To obtain this concentration in the blood stream would require the injection of an immediately fatal dose of mercurochrome-220.

Mercurochrome-220, diluted 1 : 5000 in water, killed typhoid bacilli in 5 minutes, diluted 1 : 25,000 it killed the organisms in 30 minutes and diluted in 1 : 50,000 in 1 hour. In ox bile, about 5 times as much mercurochrome-220 was required to kill the organisms; 1 : 5000 killed in 30 minutes and 1 : 10,000 killed in 1 hour.

2. Local antiseptic action on skin: It was concluded from a series of experiments made last fall that mercurochrome-220 soluble, 5% in 50% alcohol, caused no irritation of the skin and compared favorably with tincture of iodine as a skin disinfectant, against the organisms commonly present in the throat and on normal skin. Within the limitations placed

TABLE 2

BACTERICIDAL ACTION OF MERCUROCHROME-220 ON STAPHYLOCOCCUS AUREUS (C) AND ON E. TYPHI (R) (CONCENTRATIONS OF DYE REQUIRED TO KILL ORGANISMS AT VARIOUS TIME INTERVALS)

Staphylococcus aureus (C)			
Dye Solution in	15 Minutes	1 Hour	20 Hours
Distilled water	1:100	1:20,000	1:25,000
75% defibrinated blood	*	*	1:100

E. typhi (R)				
Dye Solution in	5 Minutes	30 Minutes	1 Hour	2 Hours
Distilled water	1:5000	1:25,000	1:50,000	1:50,000
Ox bile	1:1000	1:5,000	1:10,000	1:20,000

* Luxuriant growth occurred after exposure for 15 minutes, and growth also after exposure for 1 hour, to 1:100 dilution of mercurochrome-220.

by its apparent lack of action on certain spore-bearing organisms [9] it is believed that mercurochrome is useful as a local skin antiseptic.

3. Value in the treatment of various bacterial infections: Aqueous, 1% solutions of mercurochrome-220 were used for the intravenous treatment of (a) various bacterial infections of human beings, (b) experimental staphylococcus aureus septicemia of rabbits and (c) experimental typhoid cholecystitis of rabbits. The dye used was "especially prepared for intravenous use" by Hynson, Westcott and Dunning.

(a) Twelve patients, ten with genito-urinary infections, received from one to three doses of mercurochrome-220, 3 or 5 mg. per kilogram, at weekly intervals (Mendelsohn and Meddis). In the genito-urinary group were seven patients with gonorrheal infections; chronic arthritis 1, chronic prostatitis and epididymitis 2, chronic prostatitis and inguinal adenitis 1, and acute gonorrhoeal urethritis 3. This group also included two cases of chronic, and one of acute, inguinal adenitis of unknown etiology. All of these patients received the routine surgical and medical

treatment indicated, in addition to the intravenous injections of mercurochrome-220. The use of the dye had no apparent effect on gonococci or on the urethral discharge, in the cases of acute gonorrhea, nor did it contribute to the clinical recovery of any of the ten patients in this group.

One patient who had chronic empyema and pleuro-bronchial fistula with a mixed infection of the pleura and a septic type of temperature died four days after receiving one intravenous injection of mercurochrome-220 3 mg. per kilogram. This patient had been treated previously with acriviolet. One patient with septicemia due to hemolytic streptococci was given 2 doses of mercurochrome-220, 4 mg. per kilogram, 24 hours apart and died.

Intravenous doses of 5 mg. of mercurochrome-220 per kilogram given to patients produced such severe reactions that the doses were reduced to 3 mg. per kilogram. Even the latter doses often produced symptoms suggesting mercurialism, such as diarrhea, nausea, sometimes accompanied by vomiting, a temporary rise in temperature and sore gums, loosening of the teeth and salivation.

(b) When rabbits, injected intravenously with lethal amounts of Staphylococcus aureus (C), were treated immediately with one or more intravenous 5 mg. doses of mercurochrome-220, the majority of them died before the controls did. The dye did not kill the infecting staphylococci or prevent the formation of typical staphylococcic lesions in any of these animals.

(c) Following a report by Hill and Scott [11] in which they claimed that typhoid cholecystitis in rabbits could be cured by a single intravenous injection of 5 mg. of mercurochrome; and in which they recommended the use of this dye in the treatment of human typhoid carriers, Nichols carried out a series of experiments along somewhat similar lines. By means of a biliary fistula, bile was collected at intervals from a rabbit injected intravenously with 5 mg. of mercurochrome-220 per kilogram. These samples of bile were examined spectrophotometrically by French who reported that they contained mercurochrome-220 in the following concentrations: After 20 minutes the dye concentration was 1 : 16,000; 60 minutes, 1 : 2,000; 90 minutes, 1 : 3,000; 120 minutes, 1 : 5,000; 150 minutes, 1 : 6,000; 180 minutes, 1 : 7,500, and after 200 minutes, 1 : 9,000. These findings showed that mercurocrome-220 was excreted in the bile, in concentrations of from 1 : 2,000 to 1 : 9,000 for over two hours; and in view of the fact that, in vitro, the dye diluted 1 : 5,000 in ox bile killed typhoid bacilli in 30 minutes, they were considered very encouraging.

Typhoid bacilli, however, which were added to these specimens of bile containing the excreted dye, were not killed except by exposures lasting 24 hours. While these apparent discrepancies were possibly due to inherent differences between rabbit and ox bile, it is more probable that, during its passage from the blood to the bile, the mercurochrome-220 was altered chemically enough to interfere with its bactericidal action. Similar observations have been made by Hench, Snell and Green,[22] working with dogs. They found that after intravenous doses of 5 mg. of mercurochrome-220 per kilogram, the bile contained the dye in concentrations which, in vitro, should have been bactericidal for B. coli; but that it failed to show the inhibiting effect, possibly, because it was excreted in a conjugated or otherwise altered form, which greatly reduced its bactericidal action.

Nichols also used mercurochrome-220 intravenously in the treatment of typhoid cholecystitis in rabbits. The so-called typhoid "carrier condition" was produced in four of these animals by intravenous inoculations of 4 cc. of heavy salt solution suspensions of typhoid bacilli. After 12 to 28 days laparotomies were performed and bile collected from the gallbladders by means of a hypodermic syringe. Cultures were made of the bile before treatment was begun, in order to be sure that typhoid bacilli were present. Each animal was then given intravenously 5 mg. of mercurochrome-220 per kilogram on 2 successive days. When these animals were killed at the end of 4 and 17 days respectively, the gallbladders of each still contained living typhoid bacilli.

Nichols also treated one human typhoid carrier daily with doses of 100 to 200 mg. of mercurochrome-220 by mouth for one week. Medication was then discontinued because of the nausea which it caused. Cultures of the feces still contained typhoid bacilli. He concluded that mercurochrome-220 given intravenously or by mouth was of no definite value in the treatment of typhoid cholecystitis.

SUMMARY

Both acriviolet and mercurochrome-220, dissolved in water or salt solution, are effective bactericidal agents in vitro.

Both dyes in 5% solutions in 50% alcohol were found to be fairly good skin disinfectants against the normal organisms of the skin and throat.

The presence of blood reduced the in vitro bactericidal action of both dyes against staphylococci and the presence of ox bile had the same

[22] J. Pharmacol., 1925, 25, p. 142.

effect in regard to the typhoid bacilli. To obtain effective concentrations in the body would have required the intravenous injection of lethal doses of the dyes.

Acriviolet had a toxic action on normal tissues: (a) injected subcutaneously it resulted in a marked necrosis of the skin, while (b) intravenously, it caused liver and kidney injury varying in degree with the dose. Single doses of 5 mg. to 8 mg. per kilogram were followed by functional disturbances; 9 mg. per kilogram or more, by degenerative lesions which were histologically demonstrable, and 20 mg. or larger doses by death.

Acriviolet, when used intravenously in the treatment of staphylococcus septicemia in rabbits, did not kill the organisms, or prevent the formation of typical staphylococcic lesions. It did hasten the death of overwhelmingly infected animals, and it caused the death of animals infected with nonlethal amounts of staphylococci.

Acriviolet, when used intravenously in the treatment of eight patients, including six with septicemia, did not prove to be of value. Two patients were improved after treatment, but in one the focus of infection had been drained, while the other was already well on the road to spontaneous recovery, when treatment was begun. The condition of two patients was unchanged by the dye, while four became much worse and three of these died.

Intravenously, even in doses of 3 and 5 mg. per kilogram of body weight, mercurochrome-220 produced symptoms of mercurial poisoning.

Ten patients with genito-urinary infections were given mercurochrome-220 intravenously without beneficial results; and in one with septicemia due to Streptococcus hemolyticus the organisms became more numerous in the blood stream after treatment, and the patient died.

Mercurochrome-220 injected intravenously into rabbits, immediately after infection with staphylococci, did not kill the infecting organisms or prevent the formation of typical staphylococcic lesions.

Mercurochrome-220 given intravenously or by mouth failed to kill typhoid bacilli in the gallbladder of animals suffering with typhoid cholecystitis.

In view of the work here reported, it is not believed that the intravenous use of either mercurochrome-220 or acriviolet offers promise of beneficial results in the treatment of bacterial infections.

The number of clinical cases in which the dyes were used was small. Still from these results, and from the decidedly unfavorable results attending the experiments on animals, continued use of the drugs is considered unadvisable.

HEAT RESISTANCE STUDIES

2. THE PROTECTIVE EFFECT OF SODIUM CHLORIDE ON BACTERIAL SPORES HEATED IN PEA LIQUOR

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In the canning industry, it frequently happens that a process which has been yielding a sterile product for many years suddenly fails, with the result of serious losses. This laboratory has repeatedly shown that such spoilage is largely due to thermophilic bacteria whose spores have an extraordinarily high heat resistance, while in some instances it is due to putrefactive anaerobes. The classification and heat resistance studies of these organisms have been reported in earlier publications.

Since the control of this trouble entails a better knowledge of fundamental spore properties than we have at present, we have undertaken a study of the factors influencing the formation, germination and destruction of spores.

This report will be confined to study of the protective effect of common salt (sodium chloride) on bacterial spores heated in pea liquor (liquid portion of canned product). As far as we know, there has been no earlier work on this phase of spore destruction.

The multiple tube method for heating, employed in the investigation, has been described by Esty and Williams.[1] The spore suspensions also were prepared as described by them. It has been noticed that pea liquor kept in flasks was changed by oxidation to such an extent that consistent results could not be obtained. To avoid this, hand shelled peas, bought on the local market, were put up without any addition of salt or sugar as follows.

After blanching for five minutes in boiling tap water, 200 Gm. of peas were filled into no. 2 cans. The cans were filled with boiling distilled water, sealed and sterilized at 121.1 C. (250 F.) for thirty minutes. One can yielded about 300 cc. of liquor, to which the desired amount of salt could be added.

The following cultures were selected as representative of the main groups of organisms causing spoilage in canned foods:

Number.	Approximate Growth Range.	Oxygen Relations.
1	45 C.-72.5 C.	Facultative anaerobe.
2	37 C.-65 C.	Facultative anaerobe.
3	Room temperature-55 C.	Facultative anaerobe.
4	Room temperature-40 C.	Facultative anaerobe.
5	Room temperature-40 C.	Obligate anaerobe.

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[1] J. Infect. Dis., 1924, 34, p. 516.

Cultures 1, 2, 3 and 4 were incubated in the sealed thermal death point tubes where turbidity and the change in brom cresol purple were taken as criteria for growth. The indicator does not effect the heat resistance of the spores. Culture 5 was subcultured by pouring the liquor into freshly exhausted tubes of liver broth containing the ground liver tissue.

Preliminary runs were made to determine the heat resistance of the spores in the pea liquor. The time and temperature selected for each strain were such that only a small percentage of the tubes containing plain liquor were positive. These preliminary runs are summarized in

TABLE 1

HEAT RESISTANCE OF STRAINS USED IN PEA LIQUOR

Culture	1	2	3	4	5
Number of spores per cc	50,000	300,000	10,000,000	100,000	±100,000
Number of tubes each time	50	30	25	25	20
Temperature	115 C.	115 C.	105 C.	105 C.	110 C.
	% Tubes Positive for Living Spores				
20	—	60	—	100	80
21	—	—	60	—	—
22	—	3	—	—	—
24	—	3	36	—	—
25	—	—	—	100	80
27	—	—	20	—	—
30	—	—	12	100	36
33	—	—	8	—	—
35	60	—	—	100	8
36	—	—	15	—	—
40	4	—	—	88	—
45	20	—	—	88	—
50	0	—	—	2	—
55	—	—	—	4	—

The dash (—) indicates no test.

table 1. From the data in this table, the respective times and temperature for heating each strain were selected.

To determine the influence of the salt, two sets of tubes were heated. In the one set, the spores were suspended in plain pea liquor and in the other in liquor containing 1.5% sodium chloride. The results are summarized in table 2. In each case the spores were protected by the salt.

From the standpoint of the canning industry we are interested in the time necessary to kill spores. The data in table 3, gives the increase in the time necessary to kill the spores in pea liquor containing 1.5% salt. For each time there were 25 tubes with salt and 25 tubes without salt.

TABLE 2

EFFECT OF 1.5% NaCl ON HEAT RESISTANCE OF SPORES IN PEA LIQUOR

Culture	Number of Spores per Cc.	Number of Tubes in Each Set	Heating Time, Minutes	Temperature, C.	% of Tubes Positive	
					1.5% Salt	Without Salt
1	50,000	100	35	115	100	64
2	300,000	100	24	115	100	54
3	10,000,000	50	40	105	100	6
4	100,000	50	55	105	92	6
5	±100,000	50	30	110	92	0

TABLE 3

INFLUENCE OF 1.5% SALT ON THE KILLING TIME OF SPORES

Culture	1		2		3	
Temperature	115 C.		115 C.		105 C.	
	% Positive		% Positive		% Positive	
Minutes	Salt	No Salt	Salt	No Salt	Salt	No Salt
24	—	—	48	4	—	—
25	—	—	—	—	—	—
26	—	—	40	4	—	—
28	—	—	20	0	—	—
30	—	—	8	0	—	—
32	—	—	4	—	—	—
36	—	—	—	—	100	0
40	—	—	—	—	100	4
44	—	—	—	—	100	0
45	100	0	—	—	—	—
48	100	4	—	—	100	0
51	100	0	—	—	—	—
52	—	—	—	—	100	0
54	100	0	—	—	—	—
56	—	—	—	—	100	0
57	14	0	—	—	—	—
60	—	—	—	—	33	0

Same spore concentrations as in table 1.

TABLE 4

THE EFFECT OF VARIOUS CONCENTRATIONS OF SODIUM CHLORIDE ON THE HEAT RESISTANCE OF SPORES

Culture 2, 300,000 spores per cc., temperature 115 C.; 25 tubes in each set

Salt concentration, %.....	0	0.5	1	1.5	2	2.5	3	4
Positive tubes, %.....	15	37.8	86.7	73.3	75.6	78.9	40.0	13.3

With the three cultures tried, the presence of salt caused a marked increase in the time necessary to kill the spores.

The protective influence is active up to concentrations of 3 to 3.5%; 4% either shows no effect or else a killing effect. The highest protection occurs from 1 to 2.5%. Some typical results are given in table 4.

The explanation of this protective effect is not known thus far. Earlier observations seemed to indicate that sugar had the same effect and the protection was attributed to osmotic pressure, but later experi-

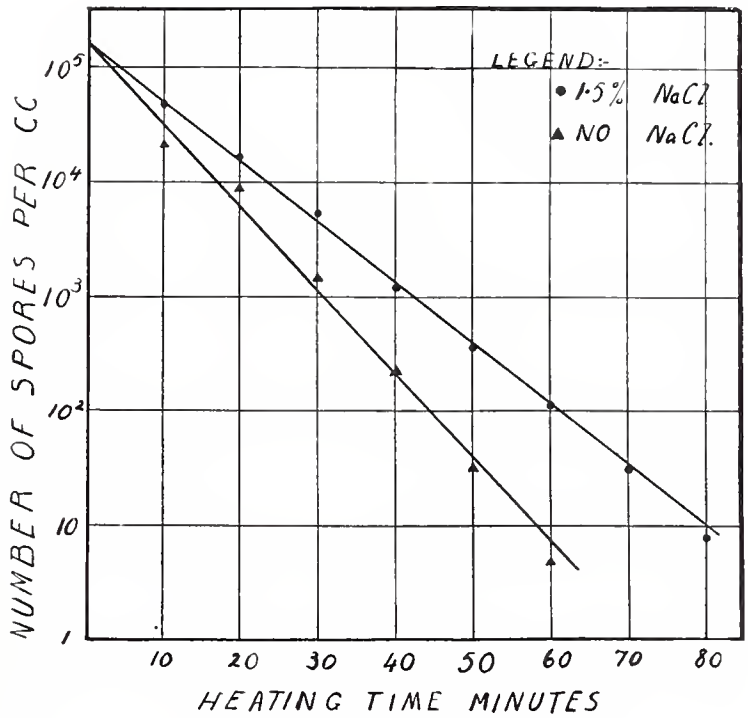

Fig. 1.—Relation of number of spores and periods of heating at 110 C., with and without sodium chloride.

obtained with equivalent molecular concentrations of sucrose from ments under proper control showed that no consistent results could be which we are lead to believe that osmosis is not the predominant factor. In a mixture of one part of pea liquor to 4 parts of distilled water the protection still occurs, which appears to be against the theory that there is a precipitation of protein around the spore, making it more impervious to water. The observation that in distilled water, even a 0.25% addition of salt exercises a killing influence suggests that the effect is dependent upon some constituent of the liquor, e. g. the protein, and the salt.

The resistance of all spores in the suspension is increased by the presence of the salt. This is borne out by the results shown in figure 1. Ten cc. portions of liquor containing 300,000 spores of culture 2 per cc. were heated at 110 C in sealed test tubes. The tubes were suspended in the oil in such a manner that the liquid could be kept in continuous agitation. At the required intervals one tube from each set was removed, cooled in ice water, and the surviving spores quantitatively plated in 0.1% dextrose yeast water agar at P_H 6.8. The number of colonies on the plate were counted after 24 hours incubation at 55 C. After every interval there were more living spores in the liquor containing the salt than in the plain liquor. The results bring out the additional fact that the spores decrease according to a logarithmic law. This law is the same as the law of unimolecular chemical reaction,

$$0.434K_1 = \frac{1}{t} \log_{10} \frac{a}{a-x}$$

This logarithmic relation in the thermal death time of spores was substantiated in an earlier publication from this laboratory.[2] Recently the same law was established by Falk and Winslow [3] for the decrease of non-spore formers in solutions of sodium chloride and calcium chloride.

SUMMARY

It has been shown that salt protects spores heated in pea liquor.

The protective effect is observed with concentration up to 4%.

The increase of time necessary to kill the spores in the presence of salt is considerable in some cases.

The resistance of all the individual spores in the suspension is increased.

Under the influence of heat the spores decrease according to the law of unimolecular chemical reaction.

[2] Bigelow, W. D.: J. Infect. Dis., 1921, 29, p. 528.
[3] J. Bact., 1926, 11, p. 1.

A STUDY OF THE VARIATION IN NUMBER OF BLOOD CELLS OF NORMAL RABBITS

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The blood of man and animals has been the subject of extensive investigations since very early times. More particularly, the cells of the blood have been investigated with reference to their number and kinds, and at the present time blood counts frequently serve as a criterion of physiologic and pathologic conditions present in the body.

Many workers have determined the average number of erythrocytes, leukocytes and the classes of leukocytes to be found in the blood of man. DaCosta,[1] Webster[2] and others have determined the extent of change in the numbers of blood cells due to normal physiologic processes and to various pathologic conditions of the human body.

Arneth[3] has classified the polymorphonuclear leukocytes of man according to the number of nuclei they contain under normal conditions and during disease.

A few investigators, Burnett,[4] and Ellerman and Bang,[5] have reported the numbers of the different kinds of blood cells in animals, although these investigators have reported the average number of erythrocytes and leukocytes and the usual percentage of the different classes of leukocytes, no extensive reports have been found of the variations which may occur under normal conditions.

Sabin, Cunningham, Doan and Kindwall[6] and Cunningham, Sabin, Sugiyama and Kindwall[7] reported on the normal variation in the blood cells of rabbits and human beings. These authors show curves of the normal distribution and variation, but did not apply the probable error to determine the limits within which these variations may be significant. There is also included an extensive bibliography on the causes of normal variations.

It is generally known that there is considerable variation in duplicate blood examinations. When a single determination is found to deviate from the normal, it is difficult to decide whether the deviation is due to normal fluctuation or to a pathologic condition of the animal body. This study was undertaken for the purpose of determining the variation in numbers of the various types of blood cells in normal rabbits. These animals were selected because they are commonly used for experimental purposes. While the results of this study are not exactly comparable to results which may be obtained from similar studies on other animals, the same laws will apply to all blood examination, and the variations will be of approximately the same relative magnitude in all cases.

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[1] Clinical Hematology, 1905.
[2] Diagnostic Methods, Chemical, Bacteriological and Microscopical, 1916.
[3] Neutrophilen Weissen Blutkorperchen bei Infektionskrankheiten, 1904.
[4] The Clinical Pathology of the Blood of Domesticated Animals, 1917.
[5] Centralbl. f. Bakteriol., I. O., 1908, 46, p. 595.
[6] Bull. Johns Hopkins Hosp., 1925, 37, p. 14.
[7] Ibid., p. 231.

METHODS OF STUDY

The normal variation in healthy rabbits was determined by making counts on a large number of individuals. The variation in the results of repeated counts on the blood of the same animal was determined by making a series of analyses at various times on the same rabbit. In order to measure the variability of the results obtained, standard deviation and probable error determinations were applied to the data. A better understanding of the variations found under normal conditions will determine the deviation necessary to indicate pathologic conditions. In order to determine to what extent personal error enters into experimental work of this kind, ten determinations were made on the same sample of citrated blood. These included total, differential and Arneth's index counts. The latter was determined according to a modified method of classification which is described later.

Total counts, differential counts and Arneth's index counts (modified) were made on a series of 100, apparently normal, healthy rabbits which had not been used for any experimental purposes. A large number of counts were also made on each of four rabbits at intervals throughout the day and on different days covering a period of several weeks.

To prevent a digestion leukocytosis, animals to be used during the day were not given their regular morning feed. All animals had access to a supply of water at all times, and so far as possible, all were subjected to the same treatment and conditions.

The total counts were made with the same hemocytometer, which had been standardized by the United States Bureau of Standards. The outfit consisted of a Levy counting chamber with Neubauer ruling, and separate pipets for the erythrocytes and leukocytes.

Blood was secured by puncturing the marginal ear vein. After wiping away the first drop, the sample of blood was collected immediately in the counting pipet and diluted at once. By doing this work as rapidly as possible, changes in the blood, due to coagulation or exposure to air were prevented.

Determination of Total Numbers.—Blood for the erythrocyte count was diluted 1 to 200 with Toisson's fluid. For the leukocyte count, blood was diluted 1 to 20 with 0.3% glacial acetic acid.

To make the ten determinations on one sample, blood was drawn from the heart of a rabbit directly into an equal volume of 1% sodium citrate solution. The pipets were filled from this citrated blood, using dilutions of 1 to 100 and 1 to 10, in the erythrocytometer and the leukocytometer respectively. Thus the same amounts of blood were examined as in the method previously described. Duplicate counts were made from each of the 10 pipets. A smear was made each time the pipets were filled for the total counts, and was used for the differential and Arneth's counts.

Preceding the making of each count, the pipet was shaken vigorously for 30 to 60 seconds. The leukocytes were allowed to settle two or three minutes in the counting chamber, and the erythrocytes five minutes before counting was begun.

After using, the counting chamber and cover glass were washed in water and wiped dry. The pipets were washed with water, alcohol and ether, and dried very carefully by means of a suction pump.

Differential Counts and Classification of Cells.—At the same time that blood was secured for the total counts, two smears were made on clean glass slides for determining the differential counts. All smears were stained by Wright's method. In making the differential counts, approximately two hundred cells were counted and classified, and their percentages determined. A mechanicad stage was used to prevent more than one observation of any one field.

The leukocytes were classified as small lymphocytes, large lymphocytes, large mononuclear leukocytes, polymorphonuclear neutrophiles, eosinophils, basophils or mast cells, and transitional cells.

Arneth's Index (Modified).—Arneth's index counts were made on the same slides used in making the differential counts. Arneth [3] separated the polymorphonuclear leukocytes into classes 1, 2, 3, 4 and 5, according to the number of separate nuclear parts each cell contained. Thus, under class 1 he included all polymorphonuclears which contained but one nucleus; under class 2, those which contained two separate and distinct nuclear parts, etc. His view was that there was a significant relationship between the age of the cell and the number of nuclear parts, the number of nuclear parts increasing with the age. Arneth obtained his index by summating the numbers of cells in classes 1 and 2 and one-half of those in class 3. He also considered that the index could be used as an aid in the diagnosis and prognosis of certain diseases, especially in tuberculosis. The more acute infections were supposed to swing the index to the "left," or, in this case, there would be a greater number of polymorphonuclear cells showing an unfragmented nucleus. These were supposed to be young leukocytes fresh from the bone marrow. Such counts have been rather widely used in the study of tuberculosis in man, and are coming into use in the study of many other diseases.

Pons and Krumbhaar [8] report the use of a modification of Arneth's system of classification. By their method, the polymorphonuclear neutrophiles are classified into three groups: metamyelocytes (very young) with round or slightly indented nuclei; nonsegmented forms (young), in which the nuclear material is connected by broad bands; segmented forms (older), in which two or more groups of nuclear material are connected by narrow filaments.

In the present study, a slight modification of Arneth's system of classification was used. The polymorphonuclear leukocytes were classified in five groups, 1, 2, 3, 4, and 5, according to the number of distinct lobes of each nucleus, as well as separated nuclear parts. The index was calculated according to Arneth's method. One hundred cells were counted in all cases.

Figure 1 shows the method of classification used. The various groups are indicated by the Roman numerals. It will be noted that the grouping in this case was made on the basis of presence of distinct nodes as well as distinct fragments of nuclear material. Such a basis for classification would give an index somewhat lower than that obtained by the original Arneth method.

It is recognized that the results of a few tests are misleading in many instances. One of the reasons for this is that in most experiments,

[8] J. Lab. & Clin. Med., 1924, 10, p. 123.

there are factors involved which cannot be controlled, even when recognized. When the most careful attempt is made to exactly reproduce a set of conditions in duplicating an experiment, it is usually found that the results vary to a greater or less extent. Therefore, the average of several tests will more nearly express the truth than any single test considered alone.

The average of several tests, however does not give the true mean. If the experiments are repeated under similar conditions, there is little probability that exactly the same results will be obtained, for the reason that it is out of the question to exactly reproduce conditions, and there may be conditions affecting the results which cannot be observed or controlled.

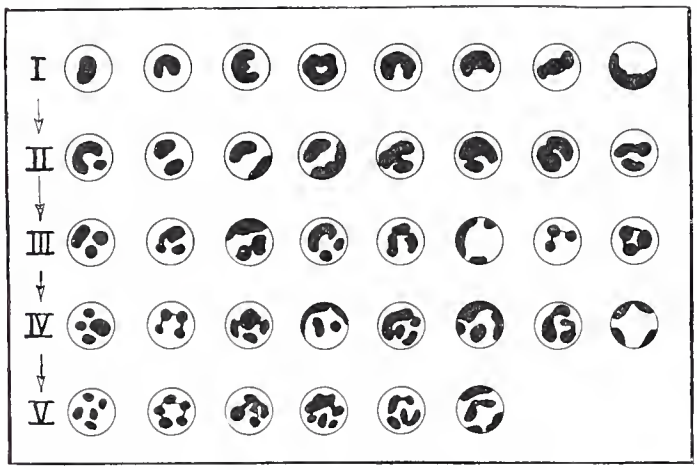

Fig. 1.—Modified Arneth's classification of polymorphonuclear leukocytes.

In performing an experiment, or a series of experiments, it is necessary to know how much reliability can be placed on the results obtained in order to interpret them in their true light, and to foretell with a fair degree of certainty, the results which can be expected from a repetition of the determination. Biometry serves as an effective method of analyzing such experimental data.

Under the study of variation, Davenport,[9] Robertson and Ray,[10] and others have shown that any set of observations tend to group themselves about a mid-value and then fall away on either side with more or less regularity.

[9] Statistical Methods, 1914.
[10] J. Biol. Chem., 1916, 24, p. 374.

The probable error concept is concerned with the fact that the average of a series of determinations does not give the true mean, and that when two sets of experiments are carried out as nearly alike as possible, the averages of the two will not be the same. By determining the probable error, figures are obtained which show the reliability that can be placed on the mean (the approximation of truth), and the probability of obtaining similar results in a similar experiment.

TABLE 1

COUNTS MADE ON THE SAME SAMPLE OF CITRATED BLOOD, with 2 DETERMINATIONS FOR EACH PIPETFUL OF BLOOD

Pipet	Erythrocytes	Leukocytes	Small Lymphocytes	Large Lymphocytes	Large Mononuclears	Polymorphonuclears	Eosinophils	Mast Cells	Transitional Cells	Arneth's Index (Modified)
1	7,872,000 7,604,000	14,800 15,200	57.5	0	0	41.0	0.5	1.0	0	58.5
2	5,226,000 5,104,000	11,200 11,400	58.0	0.5	0	39.0	1.0	1.5	0	63.0
3	6,432,000 7,824,000	12,600 12,400	54.0	1.0	0	40.5	0.5	4.0	0	55.5
4	8,280,000 7,960,000	12,400 12,200	57.0	0.5	0	40.0	1.0	1.5	0	60.0
5	6,792,000 6,368,000	12,800 14,200	59.0	1.0	0	39.0	0	1.5	0	64.0
6	7,240,000 7,200,000	12,000 12,800	60.5	0	0	39.0	0.5	1.5	0	64.0
7	6,000,000 5,128,000	12,400 12,800	54.0	1.0	0	42.0	0	3.0	0	59.0
8	6,688,000 6,232,000	11,000 11,600	52.0	2.0	0	42.0	2.0	2.0	0	65.0
9	6,328,000 5,616,000	12,000 12,600	51.0	1.0	0	45.0	1.0	2.0	0	60.0
10	6,728,000 5,876,000	11,400 11,800	52.0	2.0	1.0	40.0	0	5.0	0	58.0

The results of this study have been analyzed by the probable error concept above mentioned.

TERMS AND FORMULAS

In order to clearly understand the determination and application of probable error and standard deviation a knowledge of the formulas and meaning of the terms is essential. The definitions and formulas used in this study were obtained from the publications of Davenport,[9] Wood,[11] and others,[12] and are explained in the following paragraphs.

Modified Arneth's index determination are shown in table 3. The variates are grouped in classes under column c, and each class mean in

[11] J. Board Agric., 1911, 18, p. 630.

[12] Michael, E. L.: Science, 1920, 51, p. 89.

column x. The number of variates within each class, or the frequency, is denoted by f. Calculations were made by using the mean of the extremes of each class as the value (x in table) of each variate included within the class. Using the formulas already given, determinations were made:

	Cells per Hundred	% M (60.70)
Standard deviation (σ)	13.56	22.34
Probable error (PE)	$\pm$ 9.14	15.05
PE x 3.2	$\pm$29.24	48.17

TABLE 2

DIFFERENTIAL COUNTS FROM DIFFERENT AREAS OF THE SAME SMEAR OF BLOOD

Lymphocytes		Large Mono-nuclears	Poly-morpho-nuclears	Eosino-phils	Mast Cells	Transi-tional Cells	Arneth's Index (Modified)
Small	Large						
61.5	2.0	0	33.5	0.5	2.5	0	53.0
62.5	2.0	0	35.0	0	0	0.5	58.5
62.5	2.0	0	33.5	0.5	1.5	0	54.0
66.0	2.5	0	29.0	1.0	1.0	0.5	51.0
58.5	2.5	0	36.5	1.5	0	1.0	59.0
61.5	1.5	0	34.5	1.5	1.0	0	52.0
65.0	1.0	0	32.0	0.5	1.5	0	52.0
61.0	2.5	0	34.5	0.5	1.5	0	52.5
59.0	2.0	0	39.0	3.0	0.5	0	61.0
62.0	1.5	0	35.0	0.5	1.0	0	55.0

TABLE 3

ARNETH'S INDEX (MODIFIED)

Index in Classes c	Class Mean x	Frequency f
30.0 – 34.9	32.45	0
35.0 – 39.9	37.45	1
40.0 – 44.9	42.45	1
45.0 – 49.9	47.45	7
50.0 – 54.9	52.45	10
55.0 – 59.9	57.45	20
60.0 – 64.9	62.45	20
65.0 – 69.9	67.45	28
70.0 – 74.9	72.45	12
75.0 – 79.9	77.45	1
80.0 – 84.9	82.45	0
	Mean (M) = 60.70	

Standard Deviation.—This is an absolute measure of variability, which places additional weight on extreme variations. In a series of determinations, extreme deviations occur, which lessen the reliability of the average deviation, or the mean alone. In order to obtain a true measure of the reliability of the results, due emphasis must be placed on all the variates. This is done by averaging the square of the deviations from the mean instead of the deviations. The formula usually given for the calculation of the standard deviation is $\sigma = \sqrt{\frac{\Sigma fd^2}{N}}$, in which d stands for the deviation of each class from the mean; f, the frequency of each deviation; Σ, the summation; N, the number of variates. The standard deviation is expressed in the same terms as the magnitude of the classes. The formula used in the calculation of these data, however, is $\sigma = \frac{1}{N} \sqrt{N\Sigma fx^2 - (\Sigma fx)^2}$,

in which N is the number of variates; x, the class value; f, the frequency of each class value; and Σ, the summation. This formula was suggested by Prof. A. E. White of the mathematics department of the Kansas State Agricultural College.

TABLE 4

DAILY AND HOURLY VARIATIONS IN COUNTS FOR ONE RABBIT

Date	Time	Erythrocytes	Total Leukocytes	Small Lymphocytes, %	Polymorphonuclear Leukocytes, %	Modified Arneth's Index
6/11/23	8:45	6,536,000	12,000	60.73	33.50	62.5
	9:30	6,486,000	12,800	51.21	37.80	52.0
	10:15	6,632,000	12,600	49.19	42.24	65.0
	11:00	6,336,000	13,200	57.45	39.22	60.0
	1:15	4,568,000	13,000	51.64	44.13	65.0
	2:00	6,008,000	10,400	51.95	40.62	59.0
	2:45	5,954,000	10,800	53.41	40.59	59.0
	3:30	5,168,000	13,600	49.23	46.15	58.0
	4:15	5,048,000	11,400	59.22	36.91	57.5
6/15/23	8:45	5,136,000	10,800	54.83	35.94	53.0
	9:30	5,824,000	10,200	52.13	44.54	55.0
	10:15	6,712,000	10,600	65.02	33.33	57.0
	11:00	5,768,000	10,200	51.01	43.37	60.0
	1:15	5,056,000	10,400	52.41	44.13	57.5
	2:00	5,448,000	10,400	57.14	39.28	57.0
	2:45	5,216,000	10,200	53.65	36.17	56.0
	3:30	5,888,000	10,400	54.85	40.29	63.0
	4:15	6,720,000	10,400	53.24	38.89	62.0
6/21/23	8:45	6,976,000	10,600	62.11	29.51	63.0
	9:30	5,544,000	11,400	58.76	30.41	60.0
	10:15	5,868,000	12,600	63.08	30.37	65.0
	11:00	6,288,000	11,800	57.36	31.57	65.0
	1:15	4,616,000	11,400	47.26	37.81	62.0
	2:00	6,776,000	11,600	54.41	37.74	58.0
	2:45	6,280,000	11,400	57.36	37.05	58.0
	3:30	5,544,000	12,600	50.56	34.83	54.0
	4:15	6,462,000	11,600	55.02	37.75	54.0
7/18/23	9:45	6,112,000	10,800	55.67	33.99	51.5
	10:30	5,936,000	11,600	57.87	31.91	43.0
	11:15	5,432,000	10,600	54.46	36.62	48.0
	1:15	5,760,000	11,200	55.00	38.18	48.0
	2:00	5,884,000	10,200	52.21	38.49	48.0
	2:45	6,032,000	10,400	53.62	40.85	46.0
	3:30	5,392,000	11,200	57.45	34.04	48.0
	4:15	6,120,000	10,200	49.80	43.28	51.0
7/24/23	9:30	4,976,000	10,200	64.83	29.67	56.0
	10:15	4,616,000	10,800	61.72	31.57	53.0
	11:00	4,322,000	10,800	53.33	37.14	53.0
	1:30	4,680,000	11,000	67.90	35.11	45.0
	2:15	4,900,000	11,000	65.50	28.00	49.0
	3:00	5,032,000	10,600	50.00	42.50	53.0
	3:45	5,944,000	10,200	61.50	33.00	48.0
Extreme limits		4,322,000 to 6,976,000	10,200 to 13,600	47.26 to 67.90	28.00 to 46.15	45.0 to 65.0

Probable Error.—The probable error is arbitrarily defined as the amount that must be added to, or subtracted from, the mean of a series to obtain two limiting values within which 50% of the variates will be found. The probable error is, therefore, a measure of the confidence which may be placed in data providing the variations are due to chance and not to avoidable mistakes. The formula for calculating the probable error of a single determination is $PE = \pm 0.6745 \times \sigma$, in which PE is the probable error of a single determination and σ is the standard deviation.

In biological experiments, a normality range in which only 50% of the variates lie, is not considered sufficient margin to insure certainty that the limits of normal determinations have been reached and that everything without those limits is abnormal. To insure such certainty, the probable error is usually multiplied by 3.2. This product establishes limits within which approximately 97% of the variates lie. In other words there is about a 30 to 1 chance that a single determination will fall within the limits established by the mean plus or minus 3.2 times the probable error. It is arbitrarily assumed that if a determination falls outsides of these limits it is reasonably certain (30 to 1 chance) that the determination has been affected by some external agency.

For example, as a result of a series of one hundred leukocyte counts on normal rabbits, the mean was found to be 10,600, and the probable error ± 1,500. A single determination on a normal animal had a 1 to 1 chance of falling within the limits, 10,600 ± 1,500. When the probable error (± 1,500) was multiplied by 3.2, the limits of 5,800 and 15,400 were established. Thus there is a 30 to 1 chance that any single determination on a normal rabbit will fall within the limits of 5,800 and 15,400. If it lies without these limits there is a 30 to 1 chance that the variation is not due to chance and therefore, may indicate a pathologic condition.

The standard deviation, probable error, and the probable error multiplied by 3.2, are used in the interpretation of the blood counts made on 100 rabbits with the results shown in the following pages.

DATA

Tables 1 and 2 show the results of the determinations made to ascertain the extent of the variations inherent in the method itself.

In order to ascertain the daily and hourly variations in an individual, total and differential counts were made on four different rabbits, at successive periods of time throughout one day, at intervals of five or six days. The results of these determinations on one rabbit are shown in table 4.

DISCUSSION

The counts tabulated in table 1 were made for two purposes. The first was to determine the average difference between duplicate determinations from the same pipet and second to determine the amount of variation in several determinations made on the same sample of blood. A study of the results of the total counts shows that differences do exist between duplicate determinations. In the case of the erythrocytes, the average of the differences of the ten parallel determinations is 545,600. In the leukocyte counts, the average of the differences is 520. These variations may be due to the difference in vigor and length of time of shaking pipets or to the time elapsing between counts when the cells had an opportunity to settle in the pipets, but are probably due

to the unequal distribution of the cells throughout the diluting fluid in the pipet.

If an average is taken of all the counts and this average is considered the true count, somewhat different figures are obtained. The mean of the total erythrocyte counts is then approximately 6,600,000. The average deviation from the mean is 790,000. The mean of the total leukocyte counts is approximately 12,500. The average deviation from the mean is 800. In the differential leukocyte counts the average deviattions from the mean are somewhat greater in the case of the ten different determinations than the deviations between determinations of the distribution of the cells on the same slide.

Table 3 shows that the frequency distribution of the modified Arneth's index determinations approaches the distribution represented by a normal curve.

TABLE 5

SUMMARY OF RESULTS OF P E AND σ DETERMINATIONS

Determination	Mean	Standard Deviation σ	σ (% of M)	P E	P E (% of M)	P E × 3.2	P E × 3.2 (% of M)
Erythrocytes	5,989,500	779,358	13.01	±525,676	8.77	±1,682,100	28.08
Leukocytes	10,675	2,224.15	20.83	±1,500.18	14.05	±4,800.57	44.97
Polymorpho. leukocytes	39.10	10.88	27.57	±7.34	18.77	±23.48	60.05
Small lymphocytes	53.90	11.20	20.77	±7.55	14.01	±24.16	44.82
Large lymphocytes	2.55	2.06	80.78	±1.39	54.51	±4.45	174.51
Large mononuclears	0.43	0.51	118.60	±0.34	79.07	±1.08	251.16
Eosinophils	1.12	0.84	75.00	±0.56	50.00	±1.79	159.82
Basophils	3.58	2.17	60.61	±1.46	40.78	±4.67	130.44
Transitional cells	1.07	0.90	84.11	±0.61	57.01	±1.95	182.24
Arneth's index (modified)	60.70	13.56	22.34	±9.14	15.05	±29.24	48.17

Comparatively wide limits of normality are established by the probable error and the probable error multiplied by 3.2. This fact shows that a decided deviation from the mean index must be obtained if the index is to be of any value in the diagnosis of a pathological condition. It should be emphasized that two investigators will seldom classify a series of polymorphonuclear leukocytes in exactly the same way. Cells that are said to have two nuclear parts by one investigator will be considered as having but one by another. Thus the determination of Arneth's index is largely based on personal opinion, so that it is extremely difficult to compare the index obtained by one person with that determined by another.

Table 5 presents a summary of the results of the calculations made on the data obtained by this study.

Examples of the application of the results shown in table 5 are as follows: If we wish to produce an increase in the leukocyte count of

a rabbit, and give it a treatment for that purpose, the total leukocyte count must be at least 15,400 per cmm. for us to conclude that the treatment has produced an actual increase above the possible normal limits. In case we get a count of 15,400 or higher per cmm. we have a 30 to 1 chance that the increase is due to the treatment and not to a normal variation.

If we treat a rabbit to decrease its number of erythrocytes we must obtain a count of 4,307,000 per cmm., or less, in order to conclude that the treatment has produced the decrease. If in case of treatment, we get an erythrocyte count of less than 4,307,000 per cmm. we are justified in assuming that the treatment has produced a decrease, for the odds are 30 to 1 that a count of 4,307,000 will not occur under normal conditions.

It has been found that there is no parallel between the number of total leukocytes and the percentage values of small lymphocytes and polymorphonuclear leukocytes; i. e., when the total count is high, under normal conditions, there is no corresponding decrease or increase in the percentages of either small lymphocytes or polymorphonuclear leukocytes, and vice versa.

A study of table 4, shows that wide variations occur in the determinations of the cell counts of the peripheral blood of the same rabbit, when only 45 to 60 minutes elapse between the counts. It will be noted that the counts made one day will all be consistently higher than those made another day, or there may be wide deviations between determinations made at successive intervals of time throughout the same day. The same factors which influenced the determinations made on the 100 rabbits previously discussed are also applicable to these results. The necessity for the establishment of limits of variation, such as those obtained as a result of probable error determinations, is clearly demonstrated when these hourly and daily variations are obtained.

CONCLUSIONS

We may consider the figures obtained in this experiment as standards for the purpose of indicating conditions which affect the blood counts of normal rabbits.

In subsequent determinations on the blood of rabbits, variates whose values fall within the limits set by adding to, or subtracting from, the mean of the present series the probable error of the present series multiplied by 3.2, may be considered indicative of a normal condition of that blood.

Conversely, variates whose values fall without the limits just described may be considered as indicative of abnormal conditions of the blood.

The results of the work on the erythrocytes and leukocytes are of especial value, because counts of these two kinds of cells are very often used as a means of diagnosis of pathologic or abnormal conditions which affect the blood. The determination of limits of normal variation serves as a criterion of those deviations from the mean necessary to indicate abnormal or pathologic conditions affecting the blood.

The probable errors obtained for the small lymphocytes and polymorphonuclear leukocytes can be applied to subsequent determinations, because there is less variation and less chance for error when a relatively high count is obtained. In normal bloods, small lymphocytes and polymorphonuclear leukocytes are always found, in varying quantities it is true, but are never entirely absent.

The same calculations may be applied to the modified Arneth's index determinations, as are applied to cell counts.

The probable error figures obtained for the large lymphocytes, large mononuclear leukocytes, eosinophils, basophiles and transition cells are of doubtful value. The fact that it is often impossible to find any of these types in a particular sample of blood makes it difficult to apply any limiting values, such as those described above.

A METHOD FOR THE DETECTION OF CHANGES IN GELATIN DUE TO BACTERIA

TWO PLATES

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The ability of bacteria to liquefy gelatin has long been one of the chief characteristics used in their classification. Yet the standard method for the determination of this ability, the gelatin stab method, is known to be crude and often unreliable. Attempts have been made to modify and improve the gelatin stab technic and to add to it other determinations. Most of the methods by which satisfactory results have been obtained have involved a long or difficult technic.

Outstanding is the method of Levine and Carpenter [1] which measures the increase in amino-nitrogen as well as changes in viscosity. The technic of the viscosity determinations is, however, rather difficult for the ordinary technician.

The method to be described is that of the detection of a change in composition of the gelatin due to bacteria rather than the detection of liquefaction, although thus far all liquefying organisms have responded to the new test.

The gelatin-agar medium used for the test is made as follows: In 100 cc. of distilled water are dissolved NaCl 5.0 Gm., KH_2PO_4 0.5 Gm., K_2HPO_4 1.5 Gm.

Four grams of bacto-gelatin are dissolved in 400 cc. of distilled water, and to this solution are added dextrose 0.05 Gm., bacto-peptone 0.1 Gm., beef infusion 5.0 cc.

The two solutions are poured together, heated in a steamer, and then mixed with 500 cc. of 3% washed agar. The P_H is adjusted to about 7.0. The medium is placed in tubes or flasks and sterilized in the autoclave.

Plates of the gelatin-agar medium are poured and allowed to harden. Duplicate plates are inoculated on the surface of the agar at the center so as to form a giant colony of the organism, and are incubated for two or three days at the optimum temperature of the organism. In general, incubation for 48 hours at 30 C. was found to be satisfactory for all the organisms studied.

After incubation, one plate is flooded with a 1% solution of tannic acid, while the duplicate plate is flooded with an acid solution of bichloride of mercury of the following composition: $HgCl_2$ 15 Gm., HCl (conc.) 20 cc., and water 100 cc.

If the gelatin has been changed, a clear zone will appear about the giant colony on the plate flooded with acid bichloride of mercury

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[1] J. Bact., 1923, 8, p. 297.

solution, surrounded by the cloudy precipitate of unchanged gelatin. The reaction is slow, and 15 to 30 minutes should be allowed for its completion.

The plate flooded with tannic-acid solution will present an appearance that will vary with the amount and degree of action of the bacteria on the gelatin. An organism that acts on gelatin with little or no increase in amino-nitrogen gives a white precipitate about the colony, heavier than the precipitate of gelatin throughout the rest of the plate. With some groups of organisms this precipitate is very heavy and white, with a distinct edge. If there has been considerable decomposition of the gelatin, there is a clear zone about the colony, surrounded by a distinct white ring. This reaction takes place rapidly.

The test was originally devised to detect a change in the composition of the gelatin. When the test was applied to 215 cultures of milk organisms, it was noted that all the organisms which liquefied gelatin in the stab cultures also gave positive results with the gelatin-agar plates. But there were also organisms negative in the stab and positive on the gelatin-agar plates. Some of these organisms also showed an increase in amino-nitrogen titration in a gelatin solution, while others did not. All organisms negative by the plate method were also negative in the gelatin stab and showed no increase in amino-titration in gelatin solution.

The gelatin-agar plate method, then, detects the change in the gelatin in all cases of liquefaction and, in addition, changes without liquefaction.

A careful study was made of the action of the 215 cultures of milk organisms on gelatin. Their ability to liquefy gelatin in stab cultures was tried at least six times for each organism, and they were also tested on the gelatin-agar plates. They were also grown in a gelatin solution which was made by omitting the agar from the gelatin-agar medium. The organisms were grown in the gelatin solution for one week, after which ammonia was determined by the method of Thomas [2] and the increase in amino-nitrogen by a modification of the formol titration method and by titration in alcohol as described by Harris.[3]

A modification of Sorensen's formol titration method was devised for testing for an increase in amino nitrogen, for it was found that after the organisms had been grouped by the gelatin-agar plate method they could be further subdivided on the basis of whether or not they decomposed gelatin with an increase in amino-nitrogen. The "amino-P_H"

[2] Bull. Soc. Chemique, 1912, Ser. 4, 11, p. 796.
[3] Proc. Roy. Soc., 1923, 95B, pp. 440, 500.

method, as it has been termed, has the advantages of requiring no standard acid or alkali and of using only 1 cc. of the solution to be tested.

To 1 cc. of the solution to be tested are added 5 cc. of distilled water and a drop of phenolphthalein indicator. The tube containing 1 cc. of the control is brought to a faint pink color; that is, to a P_H of about 8.4 by the addition of dilute alkali. The tubes containing solutions to be tested are then matched to the pink color of the control; that is, brought to the same P_H. Then to each tube is added 0.5 cc. of formaldehyde solution (about 37%) which has previously been made pink to phenolphthalein. If the pink color disappears from the control solution, dilute alkali is added drop by drop until the pink color reappears. The same number of drops of the alkali are then added to each of the other tubes. If the tubes are pink no increase in amino-nitrogen has taken place. The P_H of the liquid in the colorless tubes is determined colorimetrically by the addition of phenol red or brom thymol blue indicator, and this value is called the "amino-P_H number."

This method has been found very useful in detecting small increases in amino-nitrogen and also gives a rough idea of the amount of that increase. It is useful in testing clear, synthetic medium like the gelatin solution but cannot be used, of course, in colored solutions.

In order to discover whether the small amounts of peptone and beef infusion in the gelatin-agar medium made any material difference in the determinations, the organisms were grown in a medium from which both agar and gelatin had been omitted. Although good growth took place there was no perceptible increase in the amino-titration or in ammonia. A calculation of the small amount of nitrogen added in the peptone and beef infusion shows that it could not perceptibly affect the amino-titration.

The addition of this small amount of peptone and beef infusion is necessary in the case of some organisms which cannot attack purified gelatin. Beef or casein "aminoids," (biuret free), were found unsatisfactory as a simple nitrogenous food. Peptone was better; but beef infusion was, in general, the best.

Since the action of fairly strong acid will produce a change in the gelatin that may be mistaken for a positive test, a very small amount of sugar is added and the medium is well buffered. The maximum change in P_H produced in this medium by any organism was found to be 0.4 and part of this change undoubtedly came from the gelatin. Organisms which are strong acid formers and do not attack gelatin produced an even smaller change in P_H and titrable acid after incubation

for a week. To test whether the positive tests on the plates might be due to acid formed by the bacteria, solutions of acetic and lactic acid were prepared which were ten times the strength of acid theoretically possible from the dextrose. Drops of these acid solutions gave negative tests on the plates even after action for 3 days at 30 C.

On the basis of their action on gelatin, organisms may be divided into definite groups by the aid of various tests. Organisms representative of these various groups have been selected from the 215 milk organisms and data concerning them are given in the tables below.

On the basis of their action on the gelatin in the gelatin-agar plates, the organisms may be divided into positive and negative. As was stated above, organisms which are negative by this test are negative as to liquefaction of gelatin in stab cultures and show no increase in amino-titration in gelatin solution and no test for ammonia production, although definite growth takes place in all the mediums.

TABLE 1

ORGANISMS PRODUCING A RING WITH TANNIC ACID AS A PRECIPITANT

Organism Number	Gelatin Stab Liquefaction	Gelatin-Agar Plates		Gelatin Solution		
		Tannic Acid	$HgCl_2$	NH_3	Amino-P_H Number	Amino-N.
(Control)	0	0	0	0	8.4	8.2
1	+	++ ring	+++	++	6.0	49.2
2	+	++ ring	+++	++	6.0	56.0
3	+	++ ring	+++	++	6.0	52.8
4	+	++ ring	+++	++	6.2	63.8
5	+	++ ring	+++	++	6.2	50.0
6	+	++ ring	+++	++	6.0	62.4

Each plus (+) represents a clear zone of about 1 cm. in width around colony; 0 indicates no change, and Amino-N is expressed as cc. of N/10 HCl per 100 cc. of solution, in all tables.

The organisms which give positive results on the gelatin-agar plates may be subdivided according to the results of the precipitation by tannic-acid solution into those which show a clear zone about the colony, surrounded by a distinct white ring, and those which give a more or less heavy precipitate about the colony, with little or no clear zone. The bacteria which produce the white ring fall into a group which could be called actively proteolytic. They liquefy gelatin rapidly and break it down to a considerable extent so that there is a large increase in amino-titration; and they likewise break down to a great extent a more resistant protein like casein. There is a wide, clear zone in the plate flooded with mercuric chloride solution. In table 1 are shown representatives of this group.

The number of cc. of N/10 HCl per 100 cc. of medium in the amino-titrations is an expression of the normality of free alpha-amino groups, since the method of Harris titrates these groups directly. An increase in acidity by the amino-P_H method means, of course, an increase in amino-nitrogen.

The organisms which are positive on the gelatin-agar plates and do not give a white ring fall into a number of separate groups. First on the basis of gelatin liquefaction in the stab cultures, they may be divided into positive and negative. The positive group or liquefiers may be

TABLE 2

ORGANISMS POSITIVE ON GELATIN-AGAR WITHOUT RING AND POSITIVE IN GELATIN STAB

Organism Number	Gelatin Stab Liquefaction	Gelatin-Agar Plates		Gelatin Solution		
		Tannic Acid	$HgCl_2$	NH_3	Amino-P_H Number	Amino-N.
(Control)	0	0	0	0	8.4	8.2
7	+	Precipitate	+	+	6.7	26.8
8	+	Precipitate	+	+	6.6	31.2
9	+	Precipitate	+	+	6.5	30.3
10	+	Heavy precipitate	+	0	7.7	14.6
11	+	Precipitate	+	0	8.0	7.0
12	+	Precipitate	+	0	8.0	6.4

TABLE 3

ORGANISMS POSITIVE ON GELATIN-AGAR AND WITH SLIGHT LIQUEFACTION IN GELATIN STAB

Organism Number	Gelatin Stab Liquefaction	Gelatin-Agar Plates		Gelatin Solution		
		Tannic Acid	$HgCl_2$	NH_3	Amino-P_H Number	Amino-N.
(Control)	0	0	0	0	8.4	8.2
13	Slight	Precipitate	+	+	6.5	21.4
14	Slight	Precipitate	+	+	6.6	21.8
15	Slight	Precipitate	+	Slight	6.9	15.4
16	Slight	Precipitate	+	0	7.0	20.0
17	Slight	Precipitate	+	0	8.0	6.6
18	Slight	Precipitate	+	0	8.0	6.4

further subdivided into those which give a distinct liquefaction in the stab and those which give a slight and very slow liquefaction.

The good liquefiers, as shown in table 2, generally give an increased amino-titration in the gelatin solution, but may give little or none, or even a decreased titration.

The organisms which liquefy the gelatin stab slightly and slowly are shown in table 3. All these organisms are distinctly positive with the gelatin-agar plates, but might be called either plus or minus with the gelatin stabs, depending upon chance. With eight gelatin stab cultures of organism number 15, for instance, seven were negative and the eighth showed a little liquefaction after incubation for six weeks at

20 C. Fifteen of the 215 cultures were sometimes negative, sometimes slightly positive by the gelatin stab method. Here again it will be observed that while most of the organisms give an increased amino-titration, some give little or none.

Organisms which are positive on the gelatin-agar plates and negative in the stab cultures are shown in table 4. Here again some organisms show an increase in amino-titration and others do not.

Certain organisms show other characteristics on the gelatin-agar plates that may further serve in their grouping. It has been observed that with certain organisms like 10 in table 2, in the gelatin-agar plate

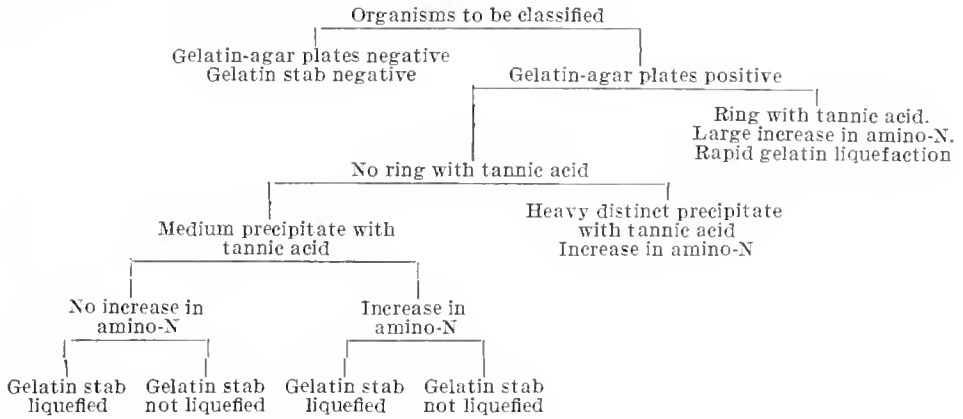
TABLE 4

ORGANISMS POSITIVE ON GELATIN-AGAR PLATES AND NEGATIVE IN GELATIN STAB

Organism Number	Gelatin Stab Liquefaction	Gelatin-Agar Plates		Gelatin Solution		
		Tannic Acid	$HgCl_2$	NH_3	Amino-p_H Number	Amino-N.
(Control)	0	0	0	0	8.4	8.2
19	0	Precipitate	+	0	7.2	15.4
20	0	Precipitate	+	+	7.0	13.0
21	0	Precipitate	+	+	7.2	10.2
22	0	Precipitate	+	0	7.0	14.6
23	0	Precipitate	+	0	8.0	5.0

flooded with tannic acid there is a very heavy, white precipitate with a distinct edge. These organisms have been classified by other methods and found to group together and to be separate from any of the other organisms. It will be noted, too, that certain organisms give a narrow, clear zone about the colony in the plate treated with tannic acid, without the formation of any ring.

A method of classification by use of the gelatin-agar plate method an amino-titration in gelatin solution is shown diagrammatically.



The gelatin stab liquefaction test has not been given a prominent place in the classification because of its slowness and unreliability. It is suggested that the test might be used, if only those organisms that showed liquefaction after a week at 20 C. were considered positive.

In table 5 are shown results obtained by the use of the gelatin-agar plate method with pure cultures of identified organisms which have been obtained from various workers. In addition to B. subtilis and B. vulgatus which are shown in the table, similar results were found with B. cohaerens, B. flavus, B. panis, B. albolactis, B. simplex, B. graveolens, B. laterosporus, B. megatherium, B. niger, B. mycoides, B. subtilis viscosus, B. adhaerens, Proteus vulgaris and B. cereus. Ps. fluorescens and Tetracoccus liquefaciens (Jensen) act like M. freudenreichii, and negative results are given by Escherichia neapolitana, B. sphaericus, L. bulgaricus and others.

TABLE 5

IDENTIFIED PURE CULTURES ON THE GELATIN-AGAR PLATES

Organism	Gelatin Stab Liquefaction	Gelatin-Agar Plates	
		Tannic Acid	$HgCl_2$
B. subtilis	+	++ ring	+++
B. vulgatus	+	++ ring	+++
B. tumescens	+	Heavy precipitate	++
B. globigii	+	Heavy precipitate	++
B. freudenreichii	+	Precipitate	+
Escherichia coli	0	0	0
Aerobacter aerogenes	0	0	0
Streptococcus lactis	0	0	0
Staphylococcus aureus	+	Precipitate	+
Aerobacter cloacae	+ slow	Weak precipitate	+

Staphylococcus aureus and Aerobacter cloacae were of special interest, because these two organisms are given as examples of bacteria which liquefy gelatin without decomposing it. This statement has been made by Mavrojannis [4] and by Berman and Rettger.[5] Yet the gelatin-agar plates indicate that some change has taken place in the composition of the gelatin about the colony. Staphylococcus aureus has been found to give a marked increase in amino-titration when grown in gelatin solution, but Aerobacter cloacae gives no increase. It is interesting to note in tables, 3, 4, and 5 that all organisms which are positive on the plates, yet give no increased amino-titration, always give, in fact, an amino-titration less than the control.

CONCLUSIONS

The gelatin-agar plate method is, then, a method by which even slight amounts of change in the composition of gelatin can be detected. The method is simple; it takes only 48 hours; incubation may be at the

[4] Ztschr. f. Hyg., 1903, 45, p. 108.
[5] J. Bact., 1918, 3, p. 367.

PLATE 1.

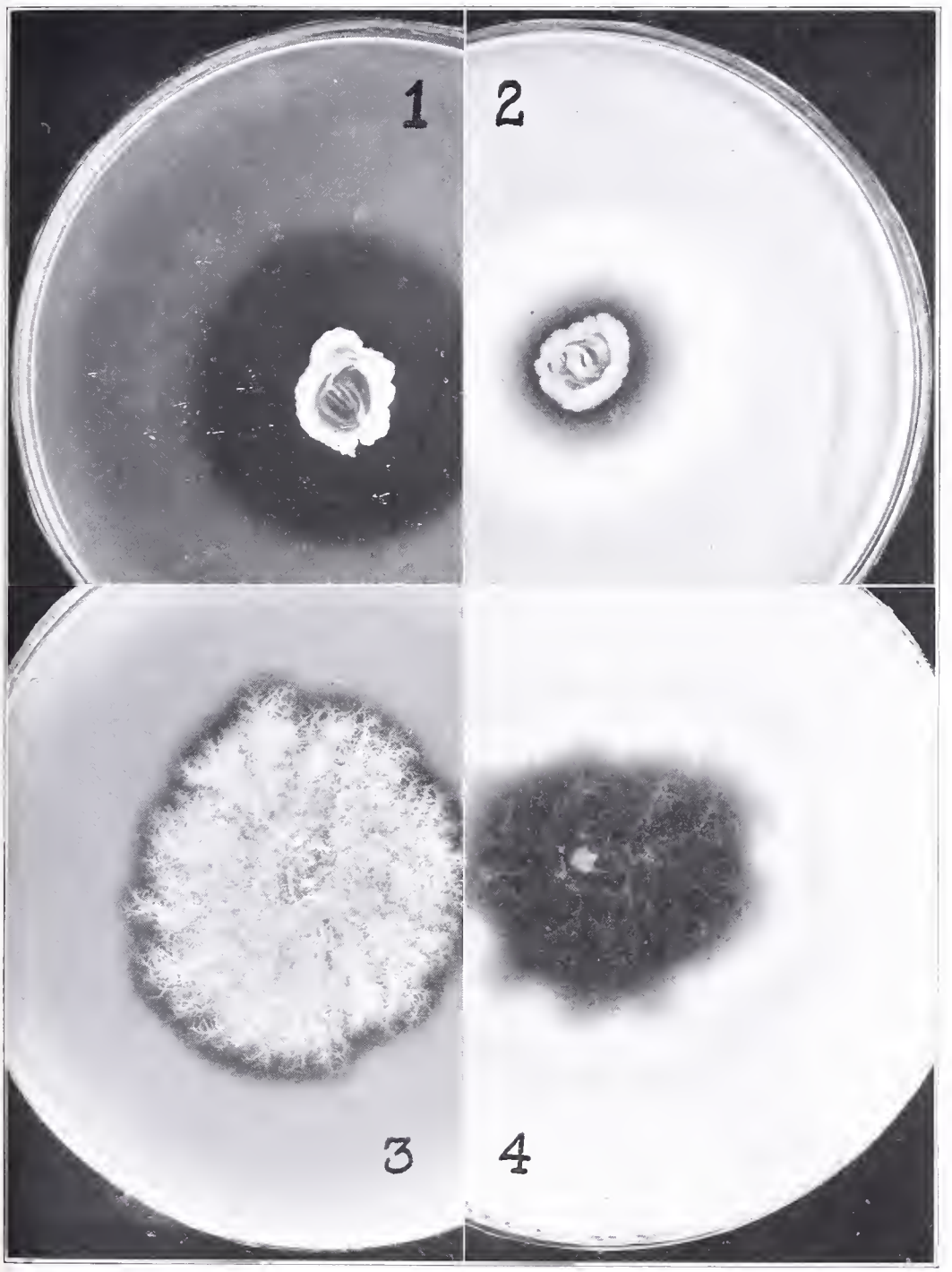


Fig. 1.—B. subtilis on gelatin-agar plate after precipitation with acid bichloride of mercury.
Fig. 2.—B. subtilis on gelatin-agar plate after precipitation with 1% tannic acid, with clear zone and white ring about colony.
Fig. 3.—B. mycoides. $HgCl_2$ as precipitant.
Fig. 4.—B. mycoides. Tannic acid as precipitant.

PLATE 2.

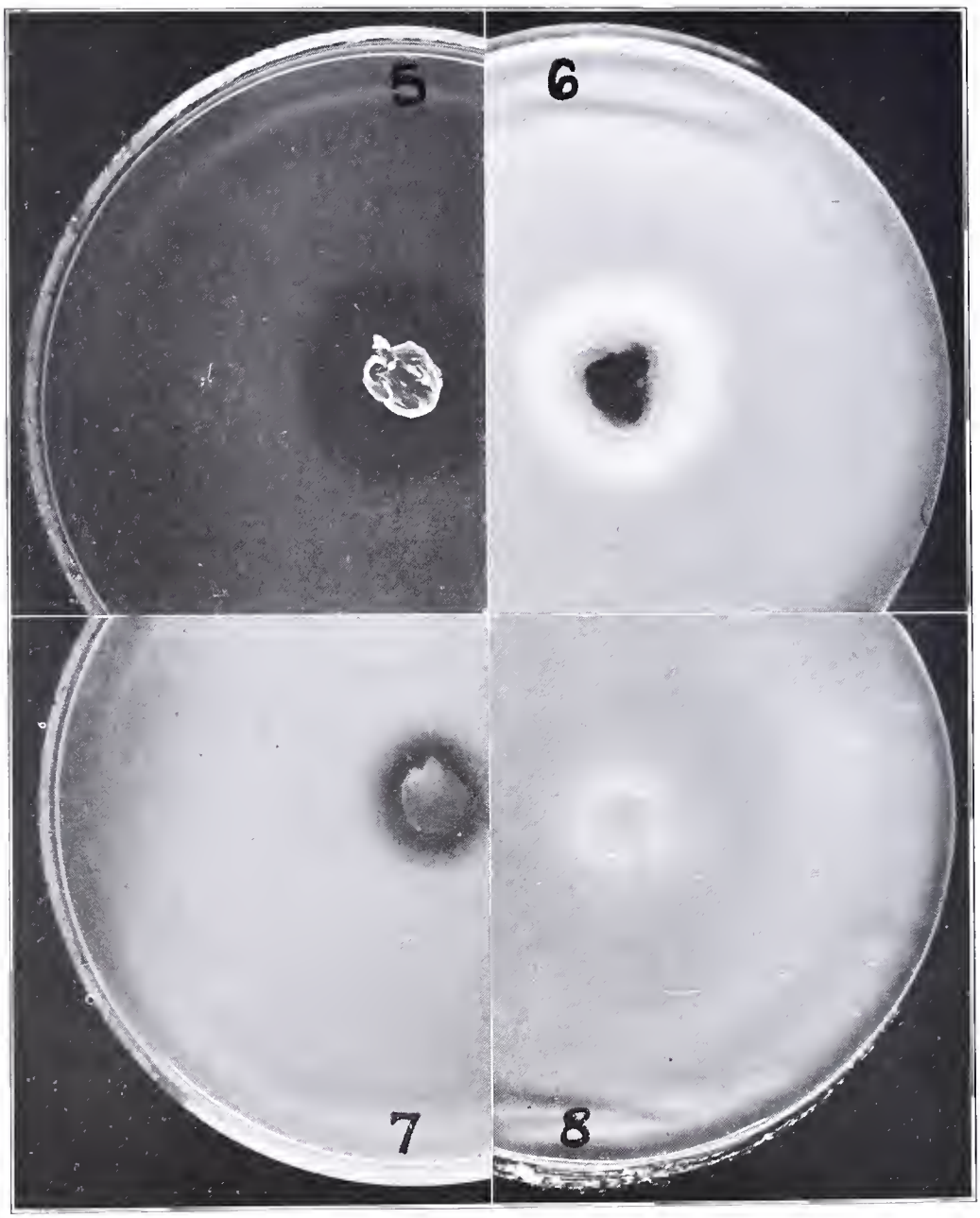


Fig. 5.—B. tumescens. $HgCl_2$ as precipitant.

Fig. 6.—B. tumescens. Tannic acid as precipitant—an example of a heavy, distinct precipitate about colony.

Fig. 7.—M. freudenreichii. $HgCl_2$ as precipitant.

Fig. 8.—M. freudenreichii. Tannic acid as precipitant.

optimum temperature of the organism; it is delicate; and in all work thus far the method has been found reliable. As with all methods it is expected that certain organisms will be found whose action is on the border line between negative and a good positive, although they have not been encountered. The method goes farther than the liquefaction tests in detecting a change in the gelatin for it includes organisms in addition to the liquefiers. It is hoped that the gelatin-agar plate method will prove useful in the classification of bacteria.

SUMMARY

The gelatin-agar plate method for the detection of a change in the composition of gelatin due to bacteria has been described. A nutrient agar is prepared with gelatin as the chief source of nitrogen. Duplicate plates of hardened gelatin-agar are so inoculated as to form giant colonies, and incubated for several days. When a plate is flooded with acid bichloride of mercury solution, a change in the gelatin due to bacteria is indicated by a clear zone about the giant colony, surrounded by precipitated gelatin. In a duplicate plate, flooded with tannic acid solution, a change is indicated by a precipitate about the colony, or with more strongly proteolytic organisms by a clear zone surrounded by a white precipitate in the form of a ring.

By the "amino-P_H" method which has been described, an increase in amino-nitrogen in a gelatin solution can be detected.

Organisms may be placed in definite groups on the basis of their action on gelatin by use of the gelatin-agar plate method and the amino-P_H method.

A NEW METHOD FOR SUSPENDING AND COUNTING LIVING TUBERCLE BACILLI

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All workers in experimental tuberculosis realize sooner or later that our present methods are inadequate for determining and controlling the dose which can infect uniformly the smaller laboratory animals. No doubt the lack of an efficient method in such experiments has hindered the accurate study of immunity in tuberculosis. Attempts have been made to immunize animals in different ways and to test their relative or absolute immunity by inoculation with varying sized doses of living organisms. The number of bacilli in these doses has been estimated in several ways, but always with a high degree of error. Of the several methods in use at the present time, one [1] depends on estimating the number of organisms per mg., moist weight, and a second on staining and counting against other bacteria or blood cells. In both these methods a high percentage of error is involved. A third method [2] is to have a constant technic for smearing and staining; and after counting the organisms in a microscopic field, to determine by animal inoculation the amount of the suspension necessary to kill an animal in a given length of time. This method does not tell how many organisms are being inoculated and has many sources of error. Barber's method,[3] in which single bacilli are drawn into capillary pipets under the microscope, is a difficult technic and would be quite inadequate for inoculating more than a few animals with a few bacilli. One is not sure, either, of the viability of the organisms on account of an appreciably long exposure to strong light.

In preparing suspensions of living tubercle bacilli for animal inoculation, in which a numerically accurate dose is required, three outstanding difficulties are encountered: that of obtaining a suspension free from clumps, of preventing spontaneous agglutination on standing, and of counting accurately the number of organisms in a unit volume of the

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[1] Calmette, A.: L'infection Bacillaire et la Tuberculose chez L'homme et chez les Aminaux, 1920, p. 243.

[2] Baldwin, E. R.: Am. Rev. Tuberc., 1921, 5, p. 429.

[3] Kansas University Sc. Bull., 1907, 4, No. 1.

suspension. The method, here presented, aims to overcome these difficulties and to avoid, at the same time, undue manipulation and exposure.

First, it seemed necessary to find a hydrogen-ion concentration at which spontaneous agglutination would not take place. Therefore, to a series of buffer solutions ranging in P_H from 4 to 9, suspensions of living tubercle bacilli were added. Maximum agglutination apparently occurred between P_H 4.5 and P_H 5, whereas clumping was absent between 8 and 9; and remained so on standing. M/15 Na_2HPO_4 proved satisfactory, as the solution is isotonic with a P_H of about 8.7.

Next a technic was attempted for preparing suspensions free of clumps. The usual method of grinding thoroughly in a mortar, suspending, allowing the larger clumps to settle out, and centrifuging the supernatant fluid never proved satisfactory, for several reasons. In the first place, there were always clumps of three to five, or more cells, and so much extraneous material was present that the eventual counting in a bacterial counting chamber was rendered almost impossible and certainly inaccurate. However, these two main objections were obviated by filtering through paper. The bacilli, taken from an asparagin synthetic medium culture, or one of glycerol broth, were blotted and ground in a mortar, in the usual way, with M/15 Na_2HPO_4 solution. The suspension was always diluted in a test tube until the outline of a pencil held against it became distinct—in other words, to about the concentration of an agglutinating suspension. This particular point seemed important for two reasons: first, a higher concentration of organisms endangered spontaneous agglutination; and second, filtration seemed perhaps most satisfactory at this dilution. Many different filter papers were tried, some being too tight and some too porous, the latter, of course, permitting clumps to pass. Whatman No. 5 was found most suitable. Two sheets were used to guard against imperfections, assuming that imperfections would not superimpose. The suspension was filtered twice directly after diluting, before the larger clumps had time to settle out, but not before the filter paper had been very thoroughly washed with distilled water to get rid of dust, shreds, etc. The resulting filtrate, after this procedure, was practically free of any foreign material and, with almost absolute uniformity, only single organisms were present. If clumps of three or more were found, the suspension was discarded. It might be well to mention here that Calmette recommends triturating the bacteria with two or three drops of bile at the outset and then proceeding further with an alkaline solution,

and states that a finer suspension can be most easily prepared in this way. But in our work, the object was not to obtain a particularly fine suspension before filtering, but merely to have a reasonable number of discrete bacilli. It appeared that perhaps a certain number of clumps were necessary to block some of the larger spaces in the paper, so that only single bacteria might pass on second filtration.

The problem of counting was not difficult. A Levy-Hausser counting chamber, ground in one piece and $\frac{1}{50}$ mm. deep, was used, with an 0.18 mm. reinforced cover slip. A high dry lens magnifying 580 X made it very easy to distinguish the bacilli, provided the lighting system was altered. It was found that by removing the Abbé condenser and replacing it with a center stop, 1 or 2 mm. in diameter, the bacilli stood out very much better and could be differentiated readily from foreign particles.

EXPERIMENTAL DATA

Exper. 1.—On Aug. 6, 1925, a large loopful of human tubercle bacilli, H_{37}, was taken from the feathery growing edge of a twenty-seven day old asparagin synthetic medium culture. After blotting between filter paper, it was triturated in an agate mortar with one drop of M/15 Na_2HPO_4 (P_H 8.7 approximately) to practical dryness. This was repeated with two drops of the same solution, after which three drops were added to make a paste; then increasing amounts of solution till a heavy suspension was obtained. This was transferred to a test tube and diluted with the phosphate solution till the outline of a pencil held against it became distinct. The suspension was then filtered twice, before the clumps had time to settle out, through two sheets of no. 5 Whatman filter paper which had been previously very thoroughly washed with distilled water. The second filtrate was immediately counted in a $\frac{1}{50}$ mm. deep Levy-Hausser counting chamber, with an 0.18 mm. reinforced cover slip.

A high dry lens magnifying 580 $\times$ and a center stop, 1 or 2 mm. in diameter, in place of the Abbé condenser, were used. Nine squares, $\frac{1}{400}$ sq. mm., were counted at a time, and the average number of bacilli per cc. of suspension was found to be 3,700,000, or 3,700 per cmm. About three hours later another count was made with an average of 3,600,000 per cc. The suspension stood at room temperature during this interim and was not shaken.

There was some difficulty in filling the counting chamber without the interference of numerous bubbles. This obstacle was overcome by swabbing the cover slip and chamber with ether. Finally, the suspension was diluted, with the phosphate solution, so that ½ cc. contained 5, 10, 25, 50 and 100 organisms. One-half cc. amounts were injected subcutaneously into the groin of a series of guinea-pigs, as tabulated in table 1.

Exper. 2.—On Sept. 25, 1925, experiment 1 was repeated as nearly as possible, and the results tabulated in table 1, part 2.

TABLE 1

RESULTS OF TUBERCULIN TESTS ON GUINEA-PIGS WITH NEGATIVE SKIN REACTIONS PREVIOUS TO INOCULATION WITH KNOWN NUMBER OF ORGANISMS

Guinea-Pig Weight in Gm.	Number of Organisms Injected Subcutaneously	Size of Reactions (mm.) to Intracutaneous Tests with 5% O. T. at Different Periods After Inoculation		
		23 days	35 days	70 days
495	5	Negative	Negative	Died
440	5	Negative	Negative	15 x 15
485	5	Negative	Negative	15 x 15 atypical
435	5	Negative	Negative	18 x 18, N
525	10	Negative	?	20 x 20, N
400	10	Negative sick	Negative sick	Negative sick
460	10	5 x 5	5 x 5	15 x 18, N
515	10	15 x 15, N	20 x 20, N	Died
505	25	?	? (Pregnant)	18 x 18, N
435	25	5 x 5, N	15 x 15, N	20 x 20, N
545	25	Negative	12 x 12	18 x 18, N
405	25	15 x 15, N	15 x 15, N	15 x 15, N
540	50	5 x 5	15 x 15, N	Died
575	50	Negative	15 x 15, N	Died
490	50	Negative	Negative Sick	18 x 18, N
420	50	Negative	15 x 15, N	20 x 20, N
560	100	20 x 20, N	20 x 20, N	25 x 25, N
445	100	15 x 15, N	25 x 25, N	15 x 15 atypical
475	100	5 x 5 (Pregnant)	18 x 18, N	18 x 18, N
490	100	10 x 10, sick	15 x 15, sick	18 x 18, N

Part 2

		20 days	35 days
420	5	Negative	±
480	5	12 x 12	15 x 15, N
550	5	Negative	Negative
530	5	Negative	Died
570	10	12 x 12	±
565	10	5 x 5	12 x 12, N
530	10	12 x 12 (Pregnant)	10 x 10 atypical
430	10	5 x 5	Died
400	25	5 x 5	15 x 15, N
515	25	12 x 12	12 x 12, N
520	25	12 x 12	15 x 15, N
400	25	12 x 12	12 x 12, N
530	50	Negative Sick	Negative Sick
450	50	12 x 12	20 x 20, N
550	50	Negative (pregnant)	10 x 10, N
510	50	12 x 12	15 x 15, N
480	100	15 x 15, N	12 x 12, sick
520	100	15 x 15	20 x 20
570	100	10 x 10	15 x 15, N
520	100	15 x 15 (Pregnant)	Negative (Pregnant)

N indicates necrotic center.

SUMMARY

In this paper is presented an accurate method for suspending and counting living tubercle bacilli. The importance of such a method lies in the fact that a precise study of experimental tuberculosis can now be undertaken, with the assurance that the infecting doses, given to smaller laboratory animals, will be uniform. In the past, the methods for determining and controlling these doses have been inadequate and consequently experiments in tuberculosis immunity were hampered.

SPONTANEOUS TUBERCULOSIS IN SALT WATER FISH

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Spontaneous tuberculosis in cold blooded animals has been noted by Sibley [1] in a ringed snake (Tropidonotus natrix), by Bataillon, Dubard and Terre [2] in a carp (Cyprinus carpio), by Friedmann [3] in turtles (Chelone corticata), and by Rupprecht [4] in a frog.

The spontaneous occurrence in salt water fish of lesions associated with acidfast bacilli was first described by Alexander [5] in lupus like ulcers of a cod fish (Gadus callarias) and by Sutherland [6] in the subcutaneous tissue and organs of a halibut (Hippoglossus hippoglossus). In neither case was the organism isolated.

Through the kindness of Dr. Henry Winsor I have had the opportunity of studying a number of salt water fish which have died in the tanks of the Philadelphia Aquarium. At necropsy numerous tubercles were found in the different organs and from these organs I have isolated an acidfast bacillus. The tissue was digested with 2% solution of sodium hydroxide during from 30 minutes to 1 hour at 37 C. and the sediment was neutralized with a 6% solution of hydrochloric acid. After centrifugation, the sediment was inoculated on different culture mediums.

The tubercles were found in the spleen and liver of all of the fish examined and in the gills, kidneys, roe, pericardium and eye of some of the fish and in one case the organism was isolated from the tissue of an intestinal ulcer. Nodules have varied in size from less than 1 mm. to 8 mm. in diameter. They had a grayish white color, were elevated, sharply circumscribed, firm, and on section have presented a uniform appearance. The intestinal ulcer measured 6 mm. in diameter. The edges and sides were ragged and irregular.

Histology.—Throughout the liver circular areas of focal necrosis were seen. These bore no relationship to the vascular supply of the tissue and were not

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[1] Virchows Archiv., 1889, 116, p. 104.
[2] Compt. rend. Soc. de biol., 1897, 4, p. 446.
[3] Ztschr. f. Tuberk. u. Heilstättenw., 1903, 4, p. 439.
[4] Inaugural Dissertation, 1904.
[5] Trans. Liverpool Biol. Soc., 1913, 27, p. 219.
[6] J. Path. & Bact., 1922, 25, p. 31.

sharply circumscribed. In these areas the liver cells were replaced by large mononuclear cells which were in great part necrotic and stained a diffuse homogeneous red with eosin. The nuclei of many of these cells had disappeared or were fragmented though many nuclei appeared normal. An occasional giant cell with nuclei in the center of the cell was seen in the periphery of these necrotic areas. Cells resembling lymphocytes of mammals were not found. The liver cells adjacent to these necrotic foci were granular, swollen, with nuclei ill defined. The blood vessels throughout the tissue were very wide and in some vessels there were masses of large mononuclear cells with clear pale staining cytoplasm The columns of liver cells were irregular in outline and the capillaries filled with blood were very wide in places. Sections stained with carbol-fuchsin decolorized with 25% sulphuric acid and counterstained with methylene blue contained numerous clumps of acidfast bacilli within the necrotic areas and smaller clumps of the same organism in the intravascular masses. The slender bacilli were arranged parallel to one another. Numerous beaded bacilli as well as numerous uniformly staining bacilli were found. A number of large mononuclear cells contained acidfast bacilli in varying numbers, occasionally replacing the cytoplasm in great part. In the spleen the walls of the blood vessels were swollen, stained homogeneously with eosin and presented a hyaline appearance. The endothelial cells of the blood vessels were necrotic and the lumens of the vessels were filled with dense felted masses of acidfast bacilli and granules of yellow pigment. The splenic tissue had undergone necrosis in places. In wide areas the cells have lost their nuclei and stain deeply with eosin; no acidfast bacilli were found in these necrotic areas.

Cultures.—Cultures have been obtained from a sergeant major (Abudefduf mauritii) from 3 croakers (Micropogon undulatus) and from 2 sea bass (Centropristes striatus). Attempts to isolate the organism from the spleen and liver of goldfish (Carassius auratus) given injections intraperitoneally with the tuberculous spleen of a lane snapper (Lutjanus griseus) were unsuccessful. Attempts to isolate the organism from the spleen and liver of frogs which received intraperitoneal injections with the tuberculous spleen of a striped bass (Roccus lineatus) did not succeed.

The first culture obtained from the spleen of the sergeant major was planted on Dorset's and Petroff's mediums and kept at 18 C. to 20 C. On glycerol agar no growth occurred and none was obtained on any medium kept at 37 C. Colonies appeared, in from 14 to 18 days after inoculation, as grayish white, moist, elevated areas having an irregular margin and closely resembling in color and consistence the cultures of the avian type of B. tuberculosis. The colonies, rapidly became larger, more moist and assumed a lemon yellow color which later became a deep orange. Transplants of the primary culture made on Dorset's or Petroff's medium yielded a more rapid and more luxuriant growth and a luxuriant growth was now obtained on glycerol agar as well as on plain agar. On agar the best growth was obtained on mediums having a reaction from 0.5% to 1.5% acid with phenolphthalein. Growth is visible after five to seven days, as moist, shiny, elevated colonies which change from a lemon yellow to a deep orange color. Growth and chromogenesis are more marked on glycerol agar than on plain agar. On agar prepared with rabbit blood no hemolysis is noted. In glycerol broth and in plain broth growth is diffuse, clouding the medium, and no pellicle is formed on the surface. In gelatin, growth occurs along the line of inoculation and liquefaction does not take place. Litmus milk is acidified and coagulated. In Dunham's medium a rapid and luxuriant growth takes place but no indol is produced, while nitrates are not reduced to nitrites.

No acid or gas is produced in dextrose, levulose, galactose, mannose, saccharose, maltose, lactose, raffinose, erythrite, dulcite, rhamnose, dextrin, inulin and glycerol. Under anaerobic conditions slight growth takes place.

The chromogenic substance does not diffuse throughout the medium. It is soluble in cold or in boiling ethyl alcohol and in cold or boiling ether but is insoluble in cold or in boiling chloroform, carbon tetrachloride, xylol or toluol.

Morphology.—In smears prepared from the tubercles, stained with carbol fuchsin and decolorized with 3% hydrochloric acid alcohol or by Gabbet's method, numerous pleomorphic acidfast bacilli were found. Short, thick, uniformly staining organisms occurred in clumps while long, thin, beaded or barred bacilli were scattered more discreetly. In smears made from the cultures the organism presented the same appearance as those found in the tubercles but with fewer beaded and barred types. No involution or branching forms have been noted in cultures three months old. The organism is nonmotile and spores are not produced.

Staining.—The bacilli stain readily with carbol-fuchsin and retain the stain after two hours' exposure to 25% sulphuric acid. With 3% hydrochloric acid alcohol some decolorization was noted after two hours, though with 5% nitric acid decolorization was more marked but remained incomplete at the end of one hour. Treated with Loeffler's methylene blue the body of the organism stains faintly, has a shadowy outline and contains granules which are metachromatic and stain deeply. The organism stains with gentian violet by Gram's method.

Pathogenicity.—Four guinea-pigs inoculated subcutaneously with the spleen of the infected sergeant major were killed 56 days later and no gross pathologic changes were found. The intraperitoneal injection of a heavy suspension of the culture into a guinea-pig produced no lesions evident five weeks later, whereas after subcutaneous injection of the same amount into a guinea-pig the regional lymph nodes became enlarged and succulent. Smears made from these enlarged nodes contained numerous beaded acidfast bacilli as well as a number of large mononuclear cells which were filled with the same organisms. A rabbit given intravenous injections with the culture, was killed two months later and no gross pathologic changes are found though a rabbit that received an intraperitoneal injection at the same time, when killed two months later, showed at necropsy a fibro-caseous mass 20 mm. in diameter, which was firmly adherent to the omentum. Smears prepared from this mass contained numerous beaded and homogeneously staining acidfast bacilli. The injection of this culture into the peritoneal cavity of a rat produced no lesions evident two months later, but an occasional acidfast organism was found in the liver though not in other organs. In two mice given injections intraperitoneally no tubercles were found in any of the organs one month later, but numerous acidfast bacilli were found in the spleen, liver and lungs. Two pigeons given injections intravenously with a heavy suspension of the culture died in eighteen and nineteen days respectively. The spleen of each was found to be somewhat enlarged. The liver contained numerous acidfast bacilli whereas none was found in the spleen, kidneys and lungs. From the livers of these pigeons we have isolated an acidfast organism which grows not only at 18 C. but at 37 C. as well. The morphologic characters of the organism when grown at 18 C. and at 37 C. were the same. Growth was obtained on Dorset's medium but not on glycerol agar.

Two frogs which received injections in the dorsal lymph sac died in ten and fifteen days respectively and at the site of injection an ulcer measuring about 6 mm. in diameter had been formed. No tubercles were found in the viscera but numerous acidfast bacilli were seen in smears from the liver, spleen, kidneys and bone marrow,

and in the lungs an occasional organism was seen. The intraperitoneal injection of this culture into two frogs caused death in ten and fifteen days respectively. At necropsy dense adhesions were found about the liver of the frog which died in fifteen days and several grayish white nodules measuring less than 1 mm. in diameter were found in the livers of both frogs, but their relation to the injected microorganism is uncertain. Numerous acidfast bacilli were found in the smears from the viscera and bone marrow of these animals. Goldfish given injections intraperitoneally died in fifteen days and though no tubercles were found in the viscera, numerous acidfast bacilli were seen in smears from the liver, spleen and kidneys.

The subcutaneous injection of the tuberculous tissue of a croaker into guinea-pigs produced no gross lesions at the site of injection. Rabbits given injections intravenously or intraperitoneally with the culture isolated from this fish showed no gross lesions five weeks later. Two pigeons died in twelve and seventeen days respectively after intravenous injection of the culture, and two mice died in nineteen and thirty days respectively after intraperitoneal injections. Four goldfish with the culture given intraperitoneally died in twenty-eight days, while frogs also given cultures intraperitoneally died in fifteen and eighteen days respectively. Frogs with injections in the dorsal lymph sac died in twenty-eight days, and ulceration was noted at the site of injection. While no tubercles were found in any of the organs of the animals dying as a result of infection with this culture, smears made from the viscera showed numerous acidfast bacilli in the spleen and liver and in the frogs acidfast bacilli were found in the bone marrow and in the ulcers present at the site of injection. From the spleen of the pigeons, mice, frogs and goldfish, an acidfast bacillus having the same characteristics as the original culture was isolated. These re-isolated cultures grew on Dorsett's and Petroff's mediums when kept at 18 to 20 C., but did not grow when placed at 37 C. No growth occurred on glycerol agar, despite the fact that the culture used for infecting these animals had been grown on glycerol agar.

The subcutaneous injection of the tuberculous spleen from a lane snapper produced in one of two guinea-pigs a caseous nodule at the site of injection. Smears from this caseous material showed numerous extracellular acidfast bacilli and a number of large mononuclear cells filled with the same organisms. Cultures from this caseous material remained sterile.

Tuberculin Production.—Raymond and Ravaut [7] and Ledoux-Lebard [8] showed that Mycobacterium piscium produced a tuberculin which when injected into tuberculous guinea-pigs or into guinea-pigs previously infected with the same culture, caused a rise of temperature of 1 C. or 2 C. This tuberculin was less toxic for tuberculous guinea-pigs than tuberculin prepared from mammalian types of B. tuberculosis.

Tuberculin was prepared from cultures of the organism isolated from the fish and grown during three weeks on glycerol broth. The broth was then concentrated to one-tenth its original volume. This tuberculin caused definite redness and edema when 0.1 cc. or 0.01 cc. was injected into the dermis of tuberculous guinea-pigs. The injection of 0.1 or 0.01 cc. into the dermis of guinea-pigs previously given injections with the tissue of the fish caused slight redness and a trace of edema. A more severe reaction was obtained when guinea-pigs infected four weeks previously with 30 mg. of the culture received 0.01 cc. of this tuberculin, intracutaneously.

[7] Compt. rend. Soc. de biol., 1898, 5, p. 587.

[8] Ann. de l'Inst. Pasteur, 1900, 14, p. 535.

When guinea-pigs previously infected with cultures isolated from the fish received intracutaneously 0.01 cc. of tuberculin prepared from a human strain of B. tuberculosis, marked redness and edema were found.

Tuberculin prepared from the several strains of cultures isolated from the fish did not give a precipitin reaction when added to a potent antituberculosis (bovine) serum.

Serologic Reactions.—By means of the complement fixation reaction Furth has found that the several cultures isolated from the salt water fish acted as the same antigen and that they differed from all of the other acidfast bacilli which were studied. These observations will be published shortly.[9] Utilizing the interface reaction as well as the agglutination reaction, Mudd[10] corroborates Furth's conclusions.

Toxin Production.—To determine if a soluble toxin was produced, the organism isolated from the fish known as sergeant major was grown during three weeks on plain broth. At the end of this time the broth was filtered through a Mandler filter and quantities of filtrate varying from 5 to 10 cc. were injected intravenously into pigeons. These pigeons remained well and when killed one month later showed no gross pathologic lesions.

DISCUSSION

The organisms which have been isolated from several salt water fish differ from the one obtained by Bataillon, Dubard and Terre[2] from a mass in the ovarian region of a carp. This organism from the carp formed in broth a growth visible after 2 to 3 days as a pellicle on the surface. On agar, white creamy colonies appeared after 3 or 4 days and after 8 days bacilli were completely replaced by branched forms. Kral and Dubard[11] found that cultures would grow at 37 C. though the optimum temperature was 22 C., but when grown at 37 C. the cultures died after 4 or 5 months. Litmus milk was unchanged by this organism. The pathogenicity of the same culture was studied by Dubard[12] who concluded that it is nonpathogenic for rabbits, birds, guinea-pigs and man, and produces tubercles in frogs, lizards or carp. On the contrary Bataillon and Terre[13] found that the intraperitoneal injection of this organism into the carp did not produce tumor like masses after 3 months though acidfast bacilli were found in smears prepared from the liver and kidneys of carp killed one month after infection. Tubercles were seen in the livers of frogs which survived infection. v. Betegh[14] found that Mycobacterium piscium was nonpathogenic for salt water fish, although eels (Anguilla vulgaris) which spawn in sea water and later

[9] J. Immunol. In press.
[10] Proc. Soc. Exper. Biol. & Med., 1926, 23, p. 569.
[11] Rev. de la Tuberc., 1898, 6, p. 129.
[12] Ibid., p. 13.
[13] Compt. rend Acad. d. Sc., 1897, 124, p. 1399.
[14] Centralbl. f. Bakteriol., I, O., 1910, 53, p. 374; 1910, 54, p. 211.

live in fresh water were susceptible to infection with the organism and developed tubercles after one month.

Bertarelli and Bocchia [15] investigated the occurrence of spontaneous tuberculosis in fish sold in different markets in Italy and, as a result of their study, concluded that such lesions were rare.

The organism I have isolated from the several salt water fish differs from Mycobacterium piscium in its morphologic, cultural, biochemical and serological character as well as in its pathogenicity. In view of these differences I suggest that it be designated Mycobacterium marinum.

SUMMARY

Tubercles were found in various organs of salt water fish found dead in the tanks of the Philadelphia Aquarium and acidfast bacilli occurred within them.

Nodules found in the liver resembled, in some respects, the tubercles of mammals and masses of acidfast bacilli were found in these nodules. In the spleen, mycotic emboli were found in the blood vessels and necrosis of vessel walls and of splenic tissue occurred.

From these lesions was isolated an acidfast, chromogenic, pleomorphic bacillus which grows best at from 18 C. to 20 C., is pathogenic for pigeons, mice, gold fish and frogs but does not kill rabbits or guinea-pigs.

The cultures isolated from these salt water fish differ in antigenic characters from the avian and mammalian varieties of B. tuberculosis, from acidfast bacilli isolated from poikilothermic animals and from saprophytic acidfast bacilli.

The name Mycobacterium marinum is suggested for this organism.

[15] Centralbl. f. Bakteriol, I. O., 1910, 54, p. 385.

A HIGHLY HEAT-RESISTANT SPORULATING ANAEROBIC BACTERIUM *

CLOSTRIDIUM CALORITOLERANS, N. SP.

ONE PLATE

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In a pooled spore suspension of Cl. botulinum type A a sporulating anaerobe with a heat resistance of 510 minutes at 100 C. was encountered. At the time the observations were made two possibilities suggested themselves concerning the origin of this bacterium, accidental contamination of the stock cultures, or a spontaneous variation of type A to a nontoxic type C strain of Cl. botulinum. An incomplete preliminary taxonomic study and the circumstances leading to the detection of the heat resistant microorganism supported at first the second possibility, a spontaneous variation. The stock culture, type A, kept in beef heart peptic digest broth had been prepared from a one cell culture in 1921; it furnished the seed material for the preparation of several hundred cultures used in the heat resistance tests reported by Esty and Meyer;[1] it remained unopened in a sealed tube for 1½ years and then revealed the presence of two types of organisms. The appearance of nontoxic cultures arising from a toxic culture has been repeatedly observed by Bengtson[2] and by workers in this laboratory, and although a mutation from a type A to a type C appeared rather remote a detailed study of the sporulating anaerobe presented more than academic interest. It was subsequently recognized that the interpretation of the incomplete biochemical study was incorrect. The organism was not agglutinated by a type C antiserum and it could not be identified with the anaerobes carefully described in the publications of the Medical Research Committee,[3] Kendall, Day and Walker,[4] Hall,[5] Kahn[6] and others. For a number of years bacteria similar in morphology and physiology to the

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[1] J. Infect. Dis., 1922, 31, p. 650.
[2] Bull. Hyg. Lab. U. S. P. H. S., 1924, No. 136, p. 41.
[3] Special Report Series, 1919, No. 39.
[4] J. Infect. Dis., 1922, 30, p. 141.
[5] Ibid., p. 445.
[6] J. M. Research, 1922, 43, p. 155.

heat resistant bacterium have been encountered in the course of some studies on the anaerobic flora of the soil.[7] A collection of five pure cultures has been subjected to a comparative taxonomic study. However, this paper deals primarily with the two heat resistant strains.

DESCRIPTION OF THE ANAEROBE

Pure cultures were secured by the deep agar shake method and by successive plating in 2% sheep blood agar under anaerobic conditions. In principle the last method is similar to the procedure advocated by Zeissler.[8] Several cultures were purified by heating liver agar dilutions in deep tubes in boiling water from two to five hours. The stock cultures were kept in brain-peptone or beefheart-peptic digest mash.

Morphology.—The organism is a slender rod. On blood plates or in beefheart medium the majority of the unstained organisms measure from 8-10 microns in length and 0.5 to 0.8 microns in thickness. Short forms with rounded ends and arranged in clusters are often encountered. Chains or filaments of varying length are observed in pasty mediums such as brain, etc. (fig. 6). The long rods are frequently slightly curved. In cultures 24 to 48 hours old rods with terminal oval, round or pear shaped spores are numerous (figs. 1, 2, 3, 4, 6 and 7). The "polar caps" or the deeply stained cytoplasmic material which is invariably noted on the poles of the spores in the preparations stained by Gram's method or with thionin are characteristic of sporulated forms. The free spores which appear on the fourth day have an average diameter of 2 microns. As a rule the young vegetative forms seem definitely gram-positive (Hucker's modification), but in 48 hours old cultures many individuals have a decided gram-negative tendency or retain the gentian violet in granules. These granules are particularly common in cultures containing carbohydrates. The young bacilli are quite motile; 8 to 12 peritrichous flagella have been demonstrated by Zettnow's method. Burke's stain tinges the spores as ring forms.

Colony Form.—Depth colonies (figs. 9 and 10): In deep liver agar small, flat, transparent discs with large polar tufts predominate. To the inexperienced, these colonies resemble those of Cl. botulinum or Cl. tetanomorphum. When young cultures grown in brain medium are transferred to liver agar a small percentage of fluffy colonies may develop. When viewed under proper magnification the colony appears as a large tuft which completely submerges the small but definite central disc.

Surface colonies (fig. 8): On glucose blood agar the colonies are small, flat, grayish, rhizoid colonies showing occasionally a dark brownish center. No hemolysis is present. On rabbit blood agar a maroon discolored narrow zone surrounds the colony, which may be classified with the "Wuchsform VII" of Zeissler.[8] The growth is characteristic and distinguishes the anaerobe from Cl. tetanomorphum or Cl. tetanoides, which form small irregularly dented colonies surrounded by either a small zone of hemolyzed red cells or by a distinct ring of an α type of methemoglobin.

CULTURAL REACTIONS

Beefheart Mash.—At the end of 48 hours there is a moderate turbidity, very slight reddening of the meat and little gas production. After four to six days the supernatant fluid is clear; a few gas bubbles continue to be formed in the

[7] Meyer and Dubovsky: J. Infect. Dis., 1922, 31, p. 530.
[8] Handb. d. Microbiol. Technik, 1923, 2, p. 1000.

meat particles for one to two weeks. There is no indication of disintegration of the meat or change in color (observation for one year).

Brain Peptone Mash with Iron.—There is moderate gas production and slight grayish discoloration of the brain particles in the top layers of the tube (14 days' incubation or longer). The odor of an old culture in this medium is faintly putrefactive.

Bromcresol Purple Milk.—No change takes place even after six months' incubation.

Broth and Casein Digest Mediums.—A slight growth is shown in plain broth but it is much better in glucose broth, within the first 24 hours. More gas is formed in the glucose broth. Sedimentation and clearing may take several days. In Wolf's casein digest fluid the abundant growth is accompanied by voluminous gas production; after three months' incubation at 37 C. whitish crystals, presumably tyrosine, are present in the sediment or throughout the culture fluid adherent to the wall of the tube.

Gelatin.—No liquefaction takes place, even after eight months' incubation at 25 to 37 C.

Solidified Albumin Cube Broth.—The medium is first cloudy then completely clears on the 10th to 15th day. There is no sign of proteolysis although the egg cube becomes grayish on prolonged incubation. A bubble of gas is formed under the vaseline cap, the hydrogen ion concentration after two months is reduced from P_H 7.2 to P_H 6.2.

Loeffler's Serum.—There is moderate growth and gas production, but no digestion.

Biochemical Reactions.—Twelve carbohydrates were used in the fermentation tests. Acid and gas production was determined in the medium recommended by Kahn.[6] The glucose-consuming and the peptolytic property were tested in accordance with the procedures published by Reddish and Rettger.[9]

Fermentation Tests.—Acid and gas are produced from glucose, galactose and maltose; gas accompanied by a slight lowering of the P_H from 7.2 to 6.6 is noted in levulose. Galactose fermentation differentiates the anaerobe from Cl. tetanomorphum and Cl. tetanoides or a nontoxic Cl. botulinum type C strain. Lactose, saccharose, raffinose, inulin, salicin, mannite, inosite and glycerol are not fermented.

Glucose-Consuming Power.—Glucose is very slowly attacked by the organism. In comparson with other terminally spored anaerobes, the amount used inside of 14 days is similar to that consumed by Cl. tetani or Cl. tertium (table 1).

TABLE 1

BIOCHEMICAL REACTIONS OF CL. CALORITOLERANS IN KAHN'S TEST MEDIUM

Glucose Broth			Plain Broth	
Changes in Present Glucose, %	Changes of P_H	Time of Incubation	Sorensen Changes,* Cc.	Changes of P_H
0.82	7.3	Control	37	7.0
0.81	7.1	12 hours	37	7.0
0.76	7.0	36 hours	37	6.9
0.71	6.7	60 hours	44	6.6
0.66	6.6	7 days	46	6.5
0.65	6.4	14 days	48	6.1

* Cc. of N/20 NaOH required to neutralize 100 Cc. of culture.

[9] J. Bact., 1924, 9, p. 13.

Peptolytic Property.—The breakdown of the peptone is slow; there is a gradual but slight increase in the Sörenson figures, which are similar to those of Cl. tetani and indicate very slight peptolytic activity. Indol is not produced.

PATHOGENICITY AND SEROLOGIC RELATIONSHIP

On numerous occasions filtrates and whole cultures of the anaerobe were fed or injected subcutaneously or intraperitoneally without producing symptoms in guinea pigs, mice or rabbits.

One strain was inagglutinable but a serum produced with this organism as an antigen clumped the second strain in a dilution of 1:2500. Both antiserums failed to agglutinate three strains of Cl. tetanomorphum, Cl. botulinum type C and Cl. parabotulinum "Seddon." The agglutinable strain (second) was not sedimented by specific antiserums prepared with Cl. tetanomorphum, Cl. sporogenes, Cl. botulinum (four groups and types) but showed a slight nonspecific co-reaction in two type serums of Cl. botulinum, type A, group 1 and type B, group 4 (Schoenholz and Meyer [10]).

In order to classify the anaerobe three strains of Cl. tetanomorphum and Cl. tetanoides from the Lister Institute, Cl. tetani, Cl. sphenoides and Cl. putrificum were tested simultaneously under identical conditions on the same medium. The differences between the heat resistant anaerobe and the feebly proteolytic Cl. tetanomorphum are definite and remained constant for a period of twelve months. The organism is considered a new species for which the specific name "Cl. caloritolerans, n. sp." is herewith proposed.

HEAT RESISTANCE TESTS

Since the circumstances which lead to the discovery of the heat resistant sporulating anaerobe suggest a number of interesting problems it is deemed important to review the essential facts. In order to apply the findings of Esty and Meyer [1] on the heat resistance of anaerobic spores an extensive series of sterilization tests of vegetables artificially contaminated with spores of Cl. botulinum has been undertaken in this laboratory. Considerable difficulties have been encountered in producing regularly an abundant yield of spores with a maximum resistance. As a rule the spores intended for the large series have been tested in preliminary experiments. For a number of months a dense spore suspension of three strains when heated from one to six hours at 100 C. gave consistently positive cultures indicative of the survival of spores

[10] J. Immunol., 1925, 10, p. 1.

after 280 to 330 minutes exposure to the temperature of boiling water. Quite unexpectedly a pooled spore suspension of the three strains produced in the same medium and under identical conditions gave toxic subcultures when boiled for 360 minutes. The same suspension was tested in vegetable juices and the survival times of the spores were at least twice that found by Esty and Meyer. A second test of the spore suspension confirmed the first result; in fact positive cultures were obtained after 470 minutes exposure in boiling water. Two of the four tubes heated for 470 minutes showed evidence of growth on the fifth

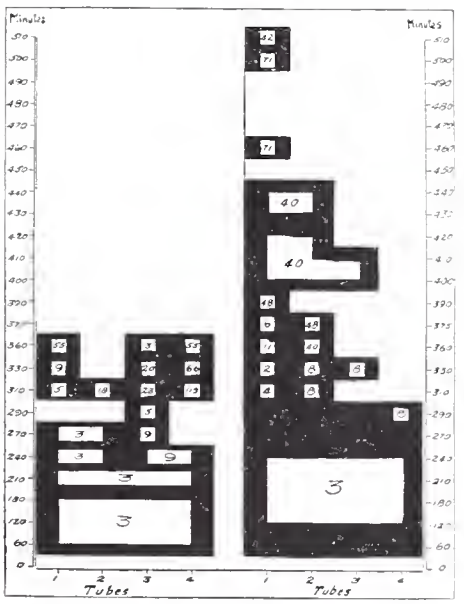


Fig. 11.—Two experiments (right and left) on growth of spores of Cl. caloritolerans after being heated at 100 C. for indicated number of minutes (ordinate figures). Four tubes (abscissa) tested each time over a period of months after heating. Shaded squares and blank squares indicate, respectively, positive and sterile tubes for period of 8 months. Numbers within spaces indicate days of dormancy before growth appearance.

day and one contained the toxin of Cl. botulinum on the 15th day of incubation. The subcultures covering the period from 370 to 470 minutes although positive were nontoxic even after prolonged incubation. It was noted that the meat particles remained unchanged. Shake cultures were then prepared and the two strains of Cl. caloritolerans were isolated.

The spore resistance of the purified strain was tested in the following manner:

Spores were secured in peptic digest—double strength—veal infusion broth incubated at 37 C. for 10 days; they were freed from the medium by centrifu-

galization, washed in sterile distilled water, suspended in 1/15 molar solution of Na_2HPO_4 and KH_2PO_4 (P_H 7.0). In order to secure uniform suspensions the mixture was mechanically shaken for several hours and strained through cheese cloth shortly before use. The number of spores in the mixture were estimated by cultivating proper dilutions in peptic digest broth. Two cc. of the suspension or approximately 20,000,000 spores were transferred to sterile pyrex tubes, which were sealed in an oxygen flame soon after they were inoculated. The content of the tubes which had been heated in boiling water at the specified times was subcultured in peptic digest beefheart—0.5% glucose—broth (P_H 7.4) and stratified with vaseline. The subcultured tubes were examined repeatedly for six months and those which showed signs of turbidity suggesting growth were studied by shake cultures. In order to detect skips and dormancy of the spores usually four tubes were removed from the water bath at the specified times. The data of two experiments are given in figure 11.

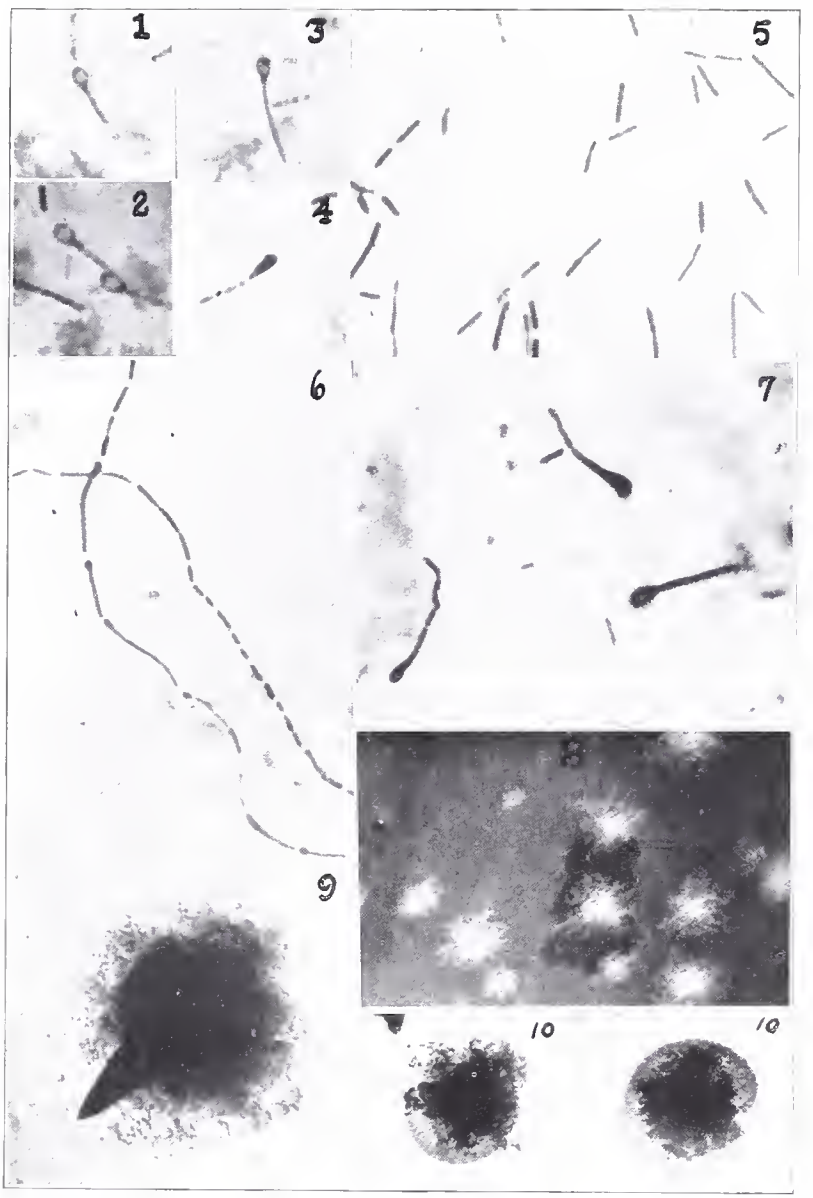
The first test with the purified strain (fig. 11) indicates that the thermal death time of the anaerobe is above 360 minutes at 100 C. In the second experiment in which tubes were heated for 720 minutes the maximum survival time was found at 520 minutes.

In both experiments above the 240 minute level the familiar occurrence of skips and delayed germination or dormancy was noted. In the first 240 minutes of the heating period the four duplicate tubes which were removed showed bacterial growth within three to nine days. Beyond that interval the prolonged dormancy and skips were more frequent; in fact the subculture at 510 minutes required an incubation period of 71 days. It is not unlikely that longer dormancy may be observed. In any case the anaerobe exhibits a behavior similar to Cl. botulinum and Cl. sporogenes with regard to retarded germination. The inference may therefore be drawn that the great majority of the spores are destroyed in the 240 minute heating interval. Beyond this time a relatively small number of spores survive and retain the ability to germinate into a new vegetative stage. The explanations given by Burke [11] for the phenomenon of dormancy is probably applicable to the retarded germination of the anaerobe described in this paper. Observations to be published elsewhere furnish circumstantial evidence which indicates that the ectoplasm of the dormant spores has a membrane of low permeability for salts and water.

Although the findings establish for the anaerobic sporulating rods a thermal death time far greater than has been previously reported, however, the data gain in significance in another direction. The numerous tests conducted by Dickson and his associates,[12] Esty and Meyer, with pure suspensions of Cl. botulinum established the death time of the

[11] J. Bact., 1923, 8, p. 555.
[12] J. Infect. Dis., 1925, 36, p. 472.

PLATE 1.



Morphology and colony types of Cl. caloritolerans.

Fig. 1.—Sporlating rod in egg albumin broth. Gram stain, × 1300.
Figs. 2, 3 and 4.—Sporulating rod in beefheart mash. Gram stam, × 1300.
Fig. 5.—Vegetative forms in casein digest broth. Gram stain, × 1300.
Figs. 6 and 7.—Vegetative and sporulating forms in brain mash. Gram stain, × 1300.
Fig. 8.—Deep colony in liver agar, × 150.
Fig. 9.—Deep colony in liver agar, × 30.
Fig. 10.—Surface colonies on sheep blood glucose-agar, × 15.

spores as 360 minutes at 100 C. The processing time of many foods preserved in tin or glass containers are based on this information. The observations detailed above indicate that the presence of other spores of greater heat resistance increase the thermal death time of Cl. botulinum from 30 to 130 minutes above the average of 360 minutes. Since the grid which acts as a vehicle for Cl. botulinum is always contaminated with other bacteria one is immediately confronted with a problem of greatest practical importance. Even if it is admitted that the finding in conjunction with the heat resistant anaerobe may be accidental, it is sufficiently definite to warrant a series of experiments which must deal with the influence of aerobic and thermophilic sporulating rods on the thermal death time of Cl. botulinum. Such tests are contemplated.

SUMMARY

This report describes the morphology, the cultural, the physiological, the serological reactions and the heat resistance of a terminally spored anaerobic bacterium for which the specific name "Clostridium caloritolerans, N. Sp." is herewith proposed.

CHARACTERISTICS OF A NEW TYPE STRAIN OF CL. TETANI

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During an investigation of human carriers of Cl. tetani in California, Bauer and Meyer [1] isolated from the stools of three individuals strains of this organism for which agglutinating serums were produced in rabbits. All were shown to belong to the same serologic group which we have tentatively considered as type 8.

One of these new type strains which was agglutinated by all three serums, 1 : 12,800, as well as two type 1 strains isolated during the same investigation were chosen for biochemical study. For purposes of comparison we have also included a type 1 strain received from the Cutter Laboratory, Berkeley, California, which they received from the U. S. Hygienic Laboratory.

In view of the conflicting description of Cl. tetani given by various authors we feel justified in recording our results even though the strains resemble one another in morphological and biochemical characters.

MORPHOLOGY

We encountered in our cultures many variations from the classical form. The differences in morphology were apparently dependent on the age of the culture and the character of the medium in which they were grown. We believe that bacilli, regardless of length or thickness, which have slightly oval end spores may be Cl. tetani. This appears to be true also for those thick forms in young cultures which sometimes show exceedingly minute round spores slightly subterminal (needle forms) or even central. Spindle forms also have been encountered. All of these forms noted in certain cultures on transference to other medium (peptic digest broth) give rise only to typical Cl. tetani. The spores of slightly oval end-sporulating organisms in young cultures tend to become perfectly round with age.

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[1] J. Infect. Dis., 1926, 38, p. 295.

Sporulation is frequently greatly delayed after the transference of cultures from agar to liquid mediums. In this case the organisms often consist entirely of very long thick filaments or short thick bacilli which to the inexperienced might be mistaken for a contaminant. The pleomorphism of Cl. tetani demands further study.

COLONY FORM

Deep Liver Agar.—During the process of purification of our strains we often noted two types of colonies. One was more or less compact and fluffy and sometimes slightly irregular in outline. The other was the round, feathery type with a dark center. Both gave rise to cultures which agglutinated with the same type serum. Usually the colony form appears to be constant in the same batch of medium for any given purified strain. No perfect discs (Hall [2]) nor discs with fluffy outgrowths (Heller [3]) were studied.

When liver agar is rather heavily seeded with pure Cl. tetani darkening of the medium occurs after several days and a black layer 1 to 4 mm. in thickness ultimately forms at the level of oxygen penetration.

CULTURAL AND BIOCHEMICAL REACTIONS

Peptic Digest Broth.—A tendency to form a massive flocculent sediment within 20-24 hours was noted in this culture medium—sporulation usually begins at this time. At the end of one month the reaction of the cultures was distinctly alkaline (P_H 7.4-7.6).

Beef Heart Mash.—All cultures resembled each other closely after two months at 37 C. The characteristics of these cultures varied greatly according to the meat used. In some instances the meat turned pink and remained so except near the upper portion which became a dirty white according to the degree of digestion. In other samples of meat there was a progressive darkening of the whole medium beginning at the edges of the meat particles near the surface. After two months the upper fourth of the meat was very dark with a blackening of the tube at the surface of the liquid. Apparently but little digestion of the meat occurred during this period but sometimes cultures several months old showed marked digestion of the upper portions of the meat. After four months there was a change in the P_H of these cultures from 7.2 to 6.8.

Brain with Iron Nail.—Distinct blackening begins at the surface within 24 hours and progresses along the nail. In two weeks the blackening of these portions is very marked.

[2] J. Infect. Dis., 1922, 30, p. 445.
[3] Ibid., p. 33.

Milk.—No gas nor any visible change was noted. The H-ion concentration remained constant during two months observation.

Solidified Egg Cubes.—The cubes are readily attacked. At the end of three months about one half the cubes ($1 \times 1 \times 1$ cm.) were disintegrated. The new type strain was the most active in this respect. The egg was darkened by all strains.

Loeffler's Serum.—At the end of three months there was no evidence of any change except marked darkening of the serum.

Gelatine Under Vaseline Cap.—All strains completely liquified gelatine in 3 to 4 days at 37 C. Fifteen days were required for complete liquefaction by the new type strain at laboratory temperature. The other strains were not tested at this temperature. No blackening of the gelatine was observed by any of them.

Hemolysis.—Hemolysis of sheep's red blood corpuscles was produced by culture filtrates of all strains. A zone of hemolysis was also noted around colonies in deep blood agar.

Indol.—Indol was produced in 5 days by all strains as well as by a second new type strain tested.

Carbohydrates.—The new type strain was tested in casein digest broth (P_H 7.2) made according to the formula cited by Kahn [4] to which the carbohydrates to be tested were added to 1% concentration. Sixteen carbohydrates were tested in this way. The P_H value of the controls after 3 to 14 days was 7.0. In the various carbohydrates tested the range changed from P_H 7.0 to 6.6 in glucose, and P_H 6.0 in one instance in xylose. The purity of these cultures was verified. A slight amount of gas was produced in most of them but after several weeks' incubation a comparison of our results with those of Kahn showed far less gas production and less increase in the H-ion concentration in our cultures than in his.

The Benedict test failed to reveal any change in starch. Several of these cultures containing carbohydrates were tested quantitatively by the colorimetric method for loss of sugar. Only in glucose was a loss noted. This occurred also with a type 1 organism. While Hall [2] and certain English workers [5] state that glucose is not fermented by Cl. tetani, Fildes [6] (for some strains) as well as Reddish and Rettger [7] believe that

[4] J. M. Research, 1922, 43, p. 155.
[5] Medical Research Committee, 1919, Series 39.
[6] Brit. J. Exper. Path., 1925, 6, p. 62.
[7] J. Bact., 1924, 9, p. 13.

this organism has slight glycolytic properties. The final determination of this question should be based upon quantitative analyses.

Toxicity.—All strains studied were toxic for mice. Four day cultures injected subcutaneously in doses of 0.01 cc. to 0.001 cc. killed mice in from 72 to 180 hours.

SUMMARY

A strain of Cl. tetani tentatively considered as type 8 has been studied comparatively with three type 1 strains. No marked differences other than serologic have been noted between these strains.

STUDY OF TETANUS AGGLUTININS AND ANTITOXIN IN HUMAN SERUMS

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A study of the bacteriology of wound infections by Tulloch[1,2] may be considered as the starting point of our newer knowledge concerning Cl. tetani. Seven serologic groups had been differentiated. Recently Bauer and Meyer[3] in this laboratory isolated from human stools three strains which we have tentatively considered to belong to a new group (type 8). We[4] have confirmed the specificity of these type strains, all of which belong to the same serologic group.

Strains of Cl. tetani were isolated from 12 human stools in England by Tulloch[1] and types 1, 2 and 3 were found to predominate in the order named. No serious studies of the types of Cl. tetani isolated from large numbers of human carriers had been undertaken until Ten Broeck and Bauer in 1921[5] and in 1924[6] reported the results of the investigations in China. They isolated and grouped 261 strains. Types 5 and 3 were found to predominate. Recently Bauer and Meyer[3] isolated Cl. tetani from 117 stool cultures (24.6% of those tested) of individuals residing in California and found that types 1 (66.6%) and 3 (18%) occurred most frequently. No mention has been made by any of these workers of the isolation of more than one type from a single stool, although Ten Broeck and Bauer[7] have reported finding agglutinins for more than one type of Cl. tetani in the serum of individual carriers and agglutinins in noncarriers.

The relation of tetanus bacilli in the digestive tract to antitoxin in the blood of residents of Pekin has received considerable attention from the last named workers. Investigating the antitoxic content of the serum of 40 Caucasian soldiers in Pekin (carrier state unknown) they found antitoxin in 37.5% of them. They further found that the serums of 26 carriers of Cl. tetani contained appreciable amounts of antitoxin and with two exceptions none was found in the serums of noncarriers, though some of these contained agglutinins. They conclude that Cl. tetani grows in the intestinal tract and sets up an immunity.

More recent investigations by Ten Broeck and Bauer[8] in which the carrier state was produced in animals revealed the fact that the serums of those animals in which Cl. tetani fails to become established showed neither agglutinins nor antitoxin. They confirmed the fact that antitoxin is not specific for type. During the process of immunization of animals agglutinins appear first and

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[1] J. Roy. Army Med. Corps, 1917,
[2] Medical Research Council, Special Report Series, 1919, No. 39, p. 29.
[3] J. Infect. Dis., 1926, 38, p. 295.
[4] Ibid., 1926, 39, p. 331.
[5] J. Exper. Med., 1922, 36, p. 261.
[6] Paper presented at the Congress of the Far Eastern Association of Tropical Medicine in Tokyo, October, 1925.
[7] J. Exper. Med., 1923, 37, p. 479.
[8] Proc. Soc. Exper. Biol. & Med., 1924, 21, p. 267.

antitoxin later. These investigators[9] have also shown that protection against subsequent infection is afforded only against the type fed to these animals. They conclude that antitoxin plays but little part in immunity to tetanus and that there is no relation between the antitoxin in the blood and resistance to infection. They believe there are other bodies specific for type which protect the animals against infection.

On the other hand Tulloch[2] believes that there is some evidence that monotypical antitoxic serums may under certain conditions exhibit specific anti-infective properties in relation to the type. He further states, with reserve, that "antibacterial serums containing no antitoxin do not prevent infection nor is there any evidence that antibacterial serums of a given antitoxin content afford more adequate protection against infection than do pure antitoxic sera of the same antitoxic content."

A careful consideration of the foregoing data and especially that supplied by Ten Broeck and Bauer will emphasize the necessity for further study concerning the relation between the carrier state and specific antibacterial immunity as well as the occurrence of agglutinins and antitoxin in the serum of carriers and noncarriers.

As the type cultures, antigens and agglutinating serums of Bauer and Meyer[3] were still available in this laboratory, the opportunity seemed excellent for a continuance of the researches of these workers. We have therefore attempted to answer the following questions:

What proportion of people residing in or near San Francisco have agglutinins to Cl. tetani in their serum? If present what types and in what dilutions do they agglutinate?

Do these type agglutinins correspond in the same proportion with the types of Cl. tetani found by Bauer and Meyer in the stools of residents of this region?

Does the serum of any of these people contain antitoxin?

Agglutination tests were made with 104 serums and of these 44 were tested for antitoxin. The technic of Ten Broeck and Bauer as given in their various publications was followed as far as practicable.

Agglutination.—We used the washed antigens of Bauer and Meyer during the early part of our work. They were frequently tested against their homologous serums and found to be potent in high dilutions. We made our own antigen for the new type strain. Type 6 was not tested as this type was not found in stools in California. The mixtures of serum and antigen (0.5 cc. each) were incubated at 37 C. for three hours. Readings were made at the end of this period and the tubes allowed to stand at room temperature for about 16 hours. They were then read again. Early in our work it became evident that the results of our agglutination tests were not in accord with the types of Cl. tetani found by Bauer and Meyer in the intestinal tract, so we made new washed antigens for types 1, 2 and 5. Type 1 antigen was made from the strain used by the Cutter Laboratory, Berkeley, California in the production of their prophylactic antiserum. The other new antigens were

[9] J. Exper. Med., 1926, 43, p. 361.

made from the type cultures previously used by Bauer and Meyer in their work. As our results continued to be the same with these new washed antigens we retested 14 serums (which previously had shown a + 1 or + 2 for type 1 at 1 : 10) comparatively with a broth antigen and the old washed antigen. No change was shown in the agglutinating titer of these serums. None of these old antigens agglutinated spontaneously and we encountered no difficulty in the reading of our results.

Toxin.—Standardized dried toxin from the U. S. Hygienic Laboratory at Washington was used. The toxin was weighed and fresh dilutions made for each series of tests.

Antitoxin Tests.—Human serum (0.1 cc.) which had been kept in the ice box only a few days was diluted 1 : 5 with saline and 0.5 cc. added to an equal amount of diluted toxin. The mixture was shaken, allowed to stand one hour at room temperature and then injected subcutaneously into white mice.

Susceptibility of White Mice.—We have found a fairly uniform reaction in these animals provided their weight does not vary more than 1-1½ grams. Each dose was invariably injected into two mice of the same approximate weight.

Controls.—In order to determine the MLD for any given serum test six to eight mice not varying more than ½ gm. in weight were given injections of toxin alone. The diluted toxin was placed in small tubes and allowed to stand at room temperature for one hour before being injected. In some instances two controls were inoculated with twice the estimated MLD for comparison with the smallest dose mixed with serum. One MLD was considered to be the least amount of toxin required to kill a 17 to 18 gram mouse in four days. The MLD of our toxin remained fairly constant throughout our work (0.0025 to 0.003 mg.).

RESULTS

Agglutinations.—There were no agglutinins in 21 (20%) of the 104 serums tested so that 80% of the latter showed agglutinins from 1 : 10 to 1 : 60 for one or more types. Fifty-three serums (50.9%) showed agglutinins at 1 : 20 or higher dilutions. The serum of 17 individuals (16.3%) agglutinated three types at 1 : 40 to 1 : 60. One serum agglutinated three types at 1 : 40, one, two types at the same titer and one, five types at varying dilutions including one type at 1 : 40. The new type found by Bauer and Meyer occurred four times at 1 : 10 dilution. The details of these agglutinations are shown in tables 1 and 2.

No agglutinations occurred in NaCl or rabbit serum controls. While admitting that nonspecific agglutinins in our serums may have been responsible for some of our positive results at 1 : 20 or perhaps even at 1 : 40 or higher, we have assumed that agglutinations at this latter titer are diagnostic of Cl. tetani. At these dilutions we have found in 17 serums the following types: type 1, 4 times (20%); type 2, 5 times (25%); type 5, 11 times (55%), (table 2). We are unable by this method to confirm the results of Bauer and Meyer who found 1 and 3 to be the predominating types in this as well as other regions. The reason

for this discrepancy may lie in the possibility that types 2 and 5 agglutinate more readily than types 1 and 3. We have not studied this point.

Antitoxin Tests.—None of the 44 serums tested (0.1 cc.) was able to neutralize 2 MLD of tetanus toxin. All but two of the serums from which agglutinations at 1 : 40 resulted as well as some which showed no agglutinins were tested.

A stool of one of us [10] whose private vegetable garden is known to be contaminated with spores of Cl. tetani was examined and found

TABLE 1

AGGLUTINATION OF 104 HUMAN SERUMS DILUTED 1:10 TO 1:60

Titer	Sex	Type 1	Type 2	Type 3	Type 4	Type 5	New Type	Total Positives	Positives for Each Dilution: Number	Positives for Each Dilution: %
1:10	Male	1	7	0	3	7	1	19	57	46.0
	Female	—	12	2	2	12	3	31		
	Unknown	—	4	1	0	2	—	7		
1:20	Male	—	5	—	1	5	—	11	47	37.9
	Female	2	9	—	1	17	—	29		
	Unknown	—	2	1	—	4	—	7		
1:40	Male	2	2	—	—	3	—	7	17	13.7
	Female	1	2	—	—	5	—	8		
	Unknown	—	—	—	—	2	—	2		
1:50	Male	—	1	—	—	—	—	1	2	1.6
	Female	—	—	—	—	1	—	1		
1:60	Male	1	—	—	—	—	—	1	1	0.8
	Female	—	—	—	—	—	—	—		
Total		7	44	4	7	58	4	—	124	100.0
%		5.7	35.5	3.2	5.7	46.7	3.2	—	—	100.0

TABLE 2

AGGLUTINATION OF 104 HUMAN SERUMS DILUTED 1:40 TO 1:60

Serums from	Number of Serums	Type 1	Type 2	Type 5	Total	%
Males	38	3	3	3	9	23.6
Females	57	1	2	6	9	15.7
Sex unknown	9	—	—	2	2	22.2
Total	104	4	5	11	20	—
%	—	20	25	55	100	—

negative. The serum was also studied and agglutinins for types 2 and 5 were present (1 : 20 and 1 : 10 respectively) but no antitoxin could be demonstrated.

We were able to obtain serums from only two individuals from whose stools Bauer and Meyer, some months previously, had isolated types 1 and 3. One serum contained no agglutinins and the other agglutinated type 2 at 1 : 20. Neither serum protected mice against 2 MLD of toxin.

[10] J. Infect. Dis., 1922, 31, p. 556.

These workers found that 24.6% of the stools studied in California contained Cl. tetani. This, taken in conjunction with the results obtained by Ten Broeck and Bauer as to the antitoxic content of the serum of carriers, would lead us to suppose that at least a certain proportion of the serums of individuals taken at random in this locality would contain antitoxin. In this connection it seems pertinent to call attention to the negative findings of Hamburger [11] who has never found a trace of antitoxin in the blood of normal animals or man.

While Buxton and Glenny [12] failed to find antitoxin in the blood of 500 horses, Römer [13] found it present in the blood of 30% of the cattle examined. The presence of the organism in the stools of horses appears to be variable, Lukas having found it in 16 or 17 stools studied, while McLeod, Soga and Roger (unpublished [14]) obtained only one positive result among upwards of 30 horses. If the antitoxic content of the serum of man and animals is dependent on the carrier state, it may very well be that negative findings of antitoxin in carriers are due to a very temporary sojourn of these organisms in the digestive tract. Further observations of many of these so-called carriers might show that the bacilli were not established nor growing as a permanent flora in any or all these individuals. Many factors might be involved in the permanency of this condition and consequently in the production or permanent establishment of antitoxin in the blood serum. In China where so many human serums contain antitoxin sanitary conditions which might have a bearing on this question, are known to be vastly different from those in California. Our failure to find antitoxin in the serum of Californians may be dependent upon some of these factors.

CONCLUSIONS

Of 104 human serums studied, 21 showed no agglutinins in dilutions of 1 : 10 or higher; 53 (50.9%) contained this antibody in dilutions of 1 : 20 or higher and 17 (16.3%) at 1 : 40 to 1 : 60.

Agglutinins occurring in serum diluted 1 :40 are assumed to be diagnostic for Cl. tetani. In this or higher dilutions type 5 was found 11 times (55%); type 2, 5 times (25%); and type 1, 4 times (20%).

The serum of a single individual may agglutinate as many as five types of Cl. tetani.

No antitoxin was found in serums, irrespective of the presence or absence of agglutinins.

[11] Jahrb. f. Kinderh., 1907, 65, p. 15; N. F. 65 Ergänzungsheft.
[12] Lancet, 1921, 2, p. 1109.
[13] Ztschr. f. Immunitätsforsch. u. exper. Therap., 1908, 1, p. 363.
[14] Browning: Brit. J. Surgery, 1916, 4, p. 14.

PROPHYLAXIS AND TREATMENT OF SNUFFLES

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VACCINATION AGAINST SNUFFLES

The following tests of prophylactic vaccination against snuffles in rabbits were made.

With Killed Bact. Lepisepticum and B. Bronchisepticus.—Young cultures (20-24 hours) of Bact. lepisepticum isolated from rabbits with typical snuffles were suspended in normal saline solution. The organisms were killed by heating in a water bath at 56 C. for 30 minutes. Virulent strains were injected into the mice seven days after the last injection with killed culture. The animals were kept under observation for five days. The rabbits which had received three vacine injections were protected in most cases (2 out of 3) against 4 MLD of Bact. lepisepticum, but not against 8 MLD. The rabbits which had received two injections were protected for the most part (2 out of 3) against 2 MLD. One of the three mice receiving only one injection lived after receiving 1 MLD, but 2 MLD killed all mice that had had only one injection.

In other words, the animals were but little protected against Bact. lepisepticum by vaccine injections of this strain. The protective power of injections of dead organisms of Bact. lepisepticum is weak compared with that of some other organisms.

A suspension of killed B. bronchisepticus organisms was injected into mice. All receiving two injections were protected against ten times the fatal dosage of this organism—a greater protection than was secured in the case of Bact. lepisepticum. B. bronchisepticus was isolated from the blood of all the dead animals.

Mice given injections with killed B. bronchisepticus were tested against living Bact. lepisepticum. All the mice, even those that had received three injections, died within four days, and Bact. lepisepticum was demonstrated from the blood of all of them.

Two rabbits received two vaccine injections of killed Bact. lepisepticum organisms (0.1 and 0.9 mg.) and were placed in the same cage with a rabbit showing snuffles of the Bact. lepisepticum type. One developed snuffles after one month and the other remained well until exposure to cold outside temperature. Bact. lepisepticum was demonstrated in the nasal secretion of both rabbits.

Two rabbits received two injections of killed B. bronchisepticus and were placed in a cage with snuffles rabbits of B. bronchisepticus type. One developed symptoms after three weeks and the other remained well for two months and showed no symptoms even after exposure to cold air.

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The evidence from these few experiments indicates little benefit from vaccination with killed organisms of Bact. lepisepticum and B. bronchisepticus.

With Living Bact. Lepisepticum and B. Bronchisepticus.—Two rabbits were given injections of Bact. lepisepticum. One rabbit received 24 mg. of killed and living Bact. lepisepticum. After fifteen injections the serum agglutinated this strain in a dilution of 1:320. The animal was bled after 51 days. It remained healthy, developing no symptoms of snuffles, and repeated examinations showed neither Bact. lepisepticum nor B. bronchisepticus. Sixty days after receiving the last injections it was placed in the same cage with a snuffles rabbit (Bact. lepisepticum type), symptoms developed in about ten days, and Bact. lepisepticum was isolated from the nasal secretion. The animal died three months after the last injection. Bact. lepisepticum was demonstrated from the blood, lung, exudate and sinus. The serum, taken before death, still agglutinated Bact. lepisepticum at 1:160.

The other rabbit received twelve injections of 16.5 mg. The serum had an agglutination titer for this organism of 1:160 after 48 days. Slight sneezing and discharge were noticed nearly four months later, and Bact. lepisepticum was demonstrated from the nasal secretion. The animal was very ill and died six months after the initial injection, although its serum still agglutinated Bact. lepisepticum at 1:80 to 1:160. Bact. lepisepticum was demonstrated from the blood, lungs, trachea.

The two rabbits injected with Bact. lepisepticum obtained no noticeable immunity against snuffles infection.

A rabbit having received twelve injections of 24.5 mg. of killed and living B. bronchisepticus organisms was bled after 37 days. The serum agglutinated B. bronchisepticus in a dilution of 1:2,500 and Bact. lepisepticum in 1:90. The animal was in good condition at the end of 48 days. After 58 days a slight discharge and sneezing were noted. Typical snuffles symptoms developed after 72 days. The serum still agglutinated B. bronchisepticus at 1:640 and Bact. lepisepticum at 1:10. The animal died 75 days after receiving the initial injection, and B. bronchisepticus was demonstrated from the blood, lungs, trachea, etc.

This showed that inoculation with B. bronchisepticus did not protect against snuffles.

IMMUNITY OF RABBITS RECOVERING FROM SNUFFLES

Rabbits improving or recovering from snuffles or subcutaneous abscesses were carefully observed.

One rabbit, which had shown severe symptoms for five months and from which Bact. lepisepticum was isolated, recovered to practically normal with absence of all symptoms. The serum agglutinated Bact. lepisepticum at 1:80. After continuing in good condition for about two months, it again developed symptoms and finally died from septicemia. Bact. lepisepticum was demonstrated from the sinus, blood, lungs, trachea and exudate in the pleural cavity. Other similar cases were observed. One rabbit, having a subcutaneous abscess from which Bact. lepisepticum was demonstrated in pure culture, finally developed septicemia and died.

Davis[1] observed a rabbit which recovered from a long-standing snuffles infection. After it had been in a practically normal condition for about four months, an abscess developed in the middle of the inner ear. The strain of organisms isolated was found identical with those involved in the preceding illness. Davis considers that animals are not immunized even to the bacilli of their own strains.

It appears that rabbits cannot be protected from snuffles either by vaccine injection of Bact. lepisepticum or B. bronchisepticus or by natural infection.

IMMUNE SERUMS FOR BACT. LEPISEPTICUM AND B. BRONCHISEPTICUS

Young cultures of Bact. lepisepticum or B. bronchisepticus were incubated at 37 C. for 20-24 hours. They were suspended in 0.85% salt solution and then mixed with the immune serum of the organisms. The mixtures were placed in the incubator at 37 C. for ten minutes and then injected intraperitoneally in 0.2 cc. amounts into mice. The mice were observed for five days, after which only one out of eight survived. This showed that the immune serum of Bact. lepisepticum in 0.2 cc. amounts hardly protects mice against the MLD of Bact. lepisepticum.

On the other hand, a similar experiment with B. bronchisepticus showed that 0.2 cc. of the immune serum of B. bronchisepticus protected mice against 2 MLD of B. bronchisepticus.

Effect of Immune Serum on the Leukocyte Count.—As described in a previous paper,[2] Bact. lepisepticum and B. bronchisepticus injected intravenously decrease the leukocyte count in the circulatory blood of rabbits.

A rabbit was injected intravenously with a mixture of 0.2 mg. of a suspension of killed Bact. lepisepticum and 0.1 cc. of immune serum of Bact. lepisepticum, the mixture having previously been incubated at 37 C. for twenty minutes. Similar tests were performed with B. bronchisepticus. The leukocyte count was decreased slightly after the intravenous injection of 0.2 cc. of the immune serum of Bact. lepisepticum, but returned to normal within three hours and after four to six hours was higher than normal, dropping again to normal within 24 hours. The results with other serums were quite similar.

The immune serum of Bact. lepisepticum shows no noticeable effect on the toxic action of Bact. lepisepticum for the leukocytes. The leukocyte count in the circulatory blood of a rabbit given intravenous injections

[1] J. Infect. Dis., 1917, 21, p. 314.
[2] Tanaka, A.: J. Infect. Dis., 1926, 38, p. 398.

with the immune serum for B. bronchisepticus and killed B. bronchisepticus, decreased slightly but soon returned to normal and then increased slightly. The results indicate that the serum of B. bronchisepticus possesses some protective power against the toxic action of B. bronchisepticus on the leukocytes.

PREVENTION AND TREATMENT OF SNUFFLES

As no protection against snuffles was secured with the antiserums from immunized rabbits and as rabbits recovering from snuffles or subcutaneous abscesses are not immune to snuffles, the prophylaxis of this disease must be sought by other methods.

Since lowered body resistance renders animals much more susceptible to snuffles, laboratory animals should have good food and be kept in well-ventilated uncrowded quarters, with high humidity and adequate warmth in winter. Strict quarantine and isolation of sick rabbits, prompt removal of dead animals and proper disinfection of cages are necessary precautions for keeping stock animals free from snuffles. Before being used in experimental work, newly received rabbits should be isolated for ten days to allow symptoms to develop if there is infection present.

In connection with the treatment of snuffles, one should note that the ordinary form of the disease in rabbits is a sinuitis associated with other symptoms.

If there is no obstruction in the respiratory passage, the bacteremia may sometimes be cured by intravenous injection of a germicide. If the sinuses are not attacked or if the purulent exudate is not tenacious the passages may be cleared by washing out the exudate. Gentian violet and mercurochrome-220 soluble, recommended by many workers, have great bactericidal action on Bact. lepisepticum and B. bronchisepticus. Both remedies were tried in our treatment of snuffles in various stages, both in intravenous injections and in washing out the nasal passages. Good results were not obtained in cases of severe sinusitis with heavy and purulent exudate.

A test was made of chlorine gas treatment for snuffles, using the "chlorin-gasator," with 0.02 mg. of chlorin gas per liter of air. Swabbings from the nasal passages of rabbits, both before and after treatment, were smeared over the surface of agar plates. Six rabbits were tested at different times in this manner with uniform results—the number of colonies and kind of bacteria on the agar plates made before and after treatment did not differ to any noticeable degree. Four of the six rabbits improved to a certain extent after four to seven treatments with

chlorine. Sneezing and coughing sometimes stopped or decreased, but none of the treated animals was cured.

SUMMARY

Rabbits which receive injections with Bact. lepisepticum or B. bronchisepticus develop agglutinins for the homologous organisms.

B. bronchisepticus vaccination gives slight but definite protection against infection with B. bronchisepticus but not against Bact. lepisepticum. Bact. lepisepticum vaccination gives a little protection against infection with Bact. lepisepticum.

B. bronchisepticus immune serum possesses some protective power against the toxic action of this organism on the leukocytes. The immune serum of Bact. lepisepticum has little protective power against the toxic action of the organism on the leukocytes.

Animals were not protected against snuffles by repeated injections of these organisms. These findings do not agree with those of Ferry and Hoskins [3] who report: "The disease can in a large measure be controlled with a vaccine . . . . of B. bronchisepticus, Bact. lepisepticum and Staphylococcus albus."

Rabbits which have recovered from snuffles, or have improved after severe infections (of the Bact. lepisepticum type), or have had subcutaneous abscesses, seem to possess very little, if any, resistance to subsequent snuffles infection.

Treatment with germicidal dyes, either intravenously or intranasally, has no effect on severe cases of snuffles, because the sinuses are severely affected.

Repeated inhalation of chlorine gas seems to have little influence on the course of symptoms in rabbits with snuffles, causing, perhaps, a temporary improvement, but never curing the condition.

[3] J. Lab. & Clin. Med., 1919, 5, p. 311.

THE PRECIPITIN REACTIONS OF EXTRACTS OF VARIOUS ANIMAL PARASITES

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In his review (1913) of the serum diagnosis in echinococcus disease Weinberg[1] gives a table of the results then at hand of precipitin tests of echinococcus protein. More recently Bryce, Kellaway and Williams[2] studied the precipitin reaction that may occur when the serum of patients with echinococcus disease is brought into contact with hydatid fluid. Beckwith and Scott[3] report that about one-half of the guinea-pigs which received intraperitoneal injections with the fluid from cysts of Cysticercus tenuicollis (sheep, hog) gave evidence of specific preciptin in the blood. As the guinea-pig is regarded as a poor precipitin producer it would be of interest to repeat this experiment with rabbits.

A detailed review of the work on complement fixation, intradermal and other reactions which are used chiefly for diagnostic purposes in echinococcus disease, is not indicated at this time. The results appear to be harmony with the laws that govern immune reactions generally.

This article gives results of observations on the precipitin reactions of materials from certain animal parasites, the antigenic reactions of most of which have not been studied before. The material was supplied through the kindness of the late B. H. Ransom, Chief, Zoological Division, and James F. Couch, Associate Chemist, Bureau of Animal Industry, Washington, D. C.

The dried and crushed parasitic substance was extracted with 0.9% salt solution, usually in the proportion of one part of the dried substance to 100 parts of solution. The amount of protein material in each extract was estimated by precipitating with heat, washing the precipitate with alcohol, and weighing the residue after drying; the strength of the extracts determined in this way are given in table 1. The albumin and globulin fractions of ascaris proteins were the same as Ransom, Harrison and Couch used in their work[4] on sensitization to ascaris. Rabbits were injected intravenously with increasing quantities of the solutions of parasitic material at intervals of three to four days. Five or six injections were given, beginning with one or

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[1] Kolle and Wassermann's Handbuch, 1913, 8, p. 148.
[2] Austr. J. Exper. Biol. and Med. Sc., 1924, 1, p. 77. See also Dew, Kellaway and Williams: Med. J. Australia, 1925, 1, p. 471.
[3] Am. J. Hyg., 1924, 4, p. 1.
[4] J. Agric. Research, 1924, 28, p. 577.

two cc. and increasing gradually to a final injection of 8 to 12 cc. Serum for precipitin tests was taken on the fourth or fifth day after the last injection. The tests were made by the contact or layer method and the results recorded after one hour's contact at the room temperature. The main results of the tests are illustrated in table 1 and the notations there give roughly the strengths of the precipitin serums as determined by the highest dilution of the antigen giving a definite reaction under the conditions stated. More rabbits and antigens other

TABLE 1

PRECIPITIN REACTIONS WITH EXTRACTS OF VARIOUS ANIMAL PARASITES

	Serums of Rabbits Given Injections with					
	Ascaris lumbricoides (hog)			Stephanurus dentatus (hog)	Gongylonema scutatum	Macracanthorhynchus hirudinaceus
Antigens	Extracts	Albumin	Globulin			
Ascaris lumbricoides (hog)						
Extract (0.3%)	10,000	400	...	0	0	0
Albumin (0.6%)	6,000	6,000	...	0	0	0
Globulin (.01%)	4,000	0	10,000	0	0	0
Ascaris columnaris (skunk)	3,000	200	...	0	0	0
Ascaris equina (0.3%)	500	300	...	0	0	0
Stephanurus dentatus (hog, 0.6%)	0	0	...	1,400	0	0
Strongylus edentatus horse, 0.2%)	0	0	...	500	0	0
Toxocara canis (dog, 0.4%)	0	200	...	0	0	0
Setaria equina (horse, 0.4%)	0	200	...	0	0	0
Cysticercus bovis	0	0	0	0	0	0
T. saginata (0.8%)	0	0	0	0	0	0
Oxyuris curvula (horse, 0.4%)	0	0	0	0	0	0
Dictyocaulus filaria (sheep)	0	0	0	0	0	0
Passalurus ambiguus (rabbit 0.65%)	0	0	0	0	0	0
Oesophagostomum columbianum (sheep, 0.5%)	0	0	0	0	0	0
Gongylonema scutatum (beef)	0	0	0	0	1,000	0
Macracanthorhynchus hirudinaceus	0	0	0	0	0	400

Normal rabbit serum gave negative (0) results with all antigens.

than those shown in the table were used, but for various reasons, the main one being a lack of antigenic material, no other positive results are now available for consideration.

SUMMARY

The results of these experiments indicate, as expected, that the precipitin reactions of materials derived from animal parasites follow the general law of species-specificness. The only exception to this rule seems to be the reaction of extract of Setaria equina, which in its

classification is far removed from the ascarids, with precipitin serum against ascaris albumin. To establish definitely the interrelations of the precipitin reaction in this case requires, however, additional observations. It is of particular interest to note that all the extracts giving precipitin reactions with the serum against ascaris albumin were found by Ransom, Harrison and Couch [4] to cause skin reactions in a person sensitive to ascaris.[5] Extension of the study of animal parasites by immunologic methods is indicated. Pathogenic forms, e. g. Dibothriocephalus latus in particular, should be given attention.

[5] See also Ransom, B. H.: J. Parasitol., 1922, 9, p. 22.

CORRECTION

In the paper "A Comparison of Strains of Brucella Abortus Isolated From Man With Those From Cattle" by C. M. Carpenter in the September issue, in the eighth line from the foot of page 218 and the fourth line from the end on page 219, "6½ months" should be changed to "231 days."

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THE PURIFICATION OF BOTULINUM TOXIN *

E. Wagner Sommer, H. Sommer and K. F. Meyer

From the George Williams Hooper Foundation for Medical Research, University of California Medical School, San Francisco

This paper records the experiments which were undertaken in order to purify the botulinum toxin with the aid of colloids. It is needless to emphasize that this study is merely part of a comprehensive investigation into the nature of one of the most potent bacterial toxins. Further reports will be made as the work progresses.

Brieger and Kempner [1] were the first to attempt the purification of Cl. botulinum toxin. They precipitated the toxic material from culture fluids by adding zinc chloride solution. The zinc compound was decomposed by alkaline ammonium phosphate and the toxin finally salted out of the filtrate with the aid of ammonium sulfate. By this method practically all the toxin was recovered. However, a second purification by the same procedure resulted in a heavy loss. Even in the more recent studies by Bengston,[2] Bronfenbrenner [3] and Schübel,[4] the only method employed for the preparation of solid toxins is that of salting out by ammonium sulfate.

The adsorption of botulinum toxin on colloids was investigated by Schübel [4] who found that the culture fluids were best detoxified by the electropositive metal hydroxides (particularly ferric hydroxide), while the toxin had little affinity for the negative colloids. According to the published data no attempt has been made to recover the poison from the absorbents.

Since bacterial toxins resemble enzymes in their physico-chemical properties, particularly in their behavior towards absorbents, it appeared of interest to apply some of the methods used for the purification of enzymes which have been developed by Willstätter and his associates [5] in an extensive series of important studies. As far as a perusal of the literature indicates, similar procedures have been applied only by Karrer and co-workers [6] to the purification of the phytotoxins, ricin and crotin, but bacterial toxins have not been tried.

Preliminary experiments have shown that botulinum toxin is strongly adsorbed by colloidal aluminum hydroxide while kaolin removed little or no toxin. After considerable experimentation the following pro-

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* This study was aided by grants from the National Canners' Association.

[1] Deutsch. med. Wchnschr., 1897, 23, p. 521.

[2] Bull. Hyg. Lab. U. S. P. H. S., 1924, No. 136, p. 87.

[3] J. Am. M. A., 1922, 78, p. 1519.

[4] Arch. f. exper. Path. u. Pharmakol., 1923, 96, pp. 237, 240.

[5] Ann. Chem. & Pharmacie, 1921, 422, p. 47; 425, p. 1; 1923, 430, p. 269. Ber. d. deutsch. chem. Gesellsch., 1922, 55, p. 3601; 1926, 59, p. 1; Ztschr. f. physiol. Chem., 1921, 116, p. 53; 1922, 123, pp. 1, 181; 1923, 125, pp. 93, 132; 126, p. 143; 1924, 132, p. 181; 133, p. 193; 138, pp. 184, 216; 140, p. 203; 1925, 142, pp. 14, 217, 245, 257; 147, pp. 248, 286; 1926, 151, pp. 1, 31, 273; 152, p. 164.

[6] Ztschr. f. physiol. Chem., 1924, 135, p. 129; Helvetica Chim. Acta, 1925, 8, p. 384.

cedure was adopted for the preparation of dried botulinum toxin: The toxic material which is adsorbed from the filtered culture mediums by aluminum hydroxide is filtered and washed. The elution of the toxin from the aluminum is effected by dilute secondary ammonium phosphate. The salts are removed by dialysis and the toxic residue is concentrated to a dry powder in vacuo.

EXPERIMENTAL

Sources of Toxin.—Cl. botulinum (type A) was grown on medium consisting of either, ⅔ blood clot digest and ⅓ beef heart or, peptic digest enriched by 2% Berna peptone. The supernatant fluid of the 2-4 weeks old cultures was decanted, filtered through paper and preserved with phenol or toluol in the icebox. The toxin titer of these solutions was usually 1 : 100,000. Throughout the experiments the toxicity has been determined on white mice and the MLD is considered that amount of toxin which kills a mouse of 20 Gm. in 48 hours. The figures are only of approximate value, since no consideration was given to the individual susceptibilities of the animals. Furthermore, the test solutions were injected only in dilution ratios of 1 : 10.

The toxin fluids are always rich in volatile bases, mostly ammonia, carbon dioxide and amino acids, which are the chief metabolic end products of Cl. botulinum.[7] The content of nonvolatile substance, determined by drying a sample in vacuo, amounts to approximately 7%. The hygroscopic, brown powder secured by evaporation of the toxic filtrate was invariably atoxic. The high salt concentration probably had a deleterious action on the poison molecule. The toxic broth filtrates furthermore contain the proteolytic enzymes of the anaerobe [7] and are therefore unstable. The removal of protein split products stimulates the action of the ferments and the fluids undergoing chemical manipulations are thus constantly changing their composition as measured by their MLD content.

Aluminum Hydroxide Adsorption.—The colloidal aluminum hydroxide was prepared according to the method given by Willstätter and Kraut;[8] the preparation C was invariably used. The suspensions contained on an average 3.5 Gm. of Al_2O_3 per liter. It was customary to stir 2 liters of culture fluid with 1 to 2 liters of aluminum hydroxide for ½ to 1 hour. The suspension was collected on a Buchner funnel and thoroughly washed. As a rule the mother liquor had a toxin titer not higher than 1 : 10. From this it is evident that the toxin was well adsorbed by the aluminum hydroxide. Using the definition of Willstätter [9] it was found convenient to express the "adsorption

[7] E. Wagner, K. F. Meyer and C. C. Dozier: J. Bact., 1925, 10, p. 321.
[8] Ber. d. deutsch. chem. Gesellsch., 1923, 56, p. 1117.
[9] R. Willstätter and W. Wassermann: Ztschr. f. physiol. Chem., 1922, 123, p. 181.

value" as the number of MLD, which are adsorbed by 1 Gm. of Al_2O_3. This value varied slightly with different lots of toxin; it was approximately 40 millions. In several cases the filtrate from 2 liters of toxin with a titer of 1:100,000 proved entirely atoxic after treatment with 1.5 liters of the colloidal suspension. Filtrates and washwaters were usually discarded when of a toxicity of 1:10 or less. These figures refer to adsorptions made at P_H 7.0-7.6 without adjusting the reaction of the toxic broth. At different reactions (P_H 8.4 and 5.0) the toxin was likewise adsorbed, although complicating factors are involved, the study of which is now in progress.

Elution.—Weak acetic acid (P_H 3.8) and ammonia (P_H 8.4) were tried for elution of the toxin from the adsorbents; both proved to be unsatisfactory. Ammonium phosphate was tested in several concentrations. A solution of 0.5% was just as efficient as those of 1, 5, or 10%; this concentration was therefore chosen. Elutions were made at different reactions. The yield of the toxin was the same at P_H 3.8, 7.8 and 8.4. However, upon standing the alkaline solutions proved to be more stable; therefore secondary ammonium phosphate which gives a reaction of P_H 8.0 was used. The addition of glycerol, a slightly elevated temperature (28 C), and standing for several hours greatly increased the efficiency of the elution. The following method was finally adopted as a routine procedure. The washed aluminum mass is thoroughly triturated with ½ to 1 liter of 0.5% $(NH_4)_2HPO_4$, 0.1% glycerol is added and the mixture placed in the 28 C. incubator for 5 hours. The aluminum hydroxide is filtered off by suction and the process is repeated once or twice. In this way 30-50% of the toxin originally present is recovered in the filtrate. The different elutions are united for dialysis.

Dialysis.—Various dialysing membranes were investigated. Large collodion bags could not be prepared which permitted a rapid diffusion of the salts. (2 to 8% solutions of Parlodion Du Pont in three parts of ether and one part of alcohol with the addition of ethyl acetate were used.) Dialyzing parchment (Arthur H. Thomas No. 4463) was found to be too porous for botulinum toxin. Commercially prepared fish bladders, although slightly permeable for the toxin, proved satisfactory and were used throughout. During prolonged dialysis occasionally an atoxic precipitate formed which was removed by centrifugation.

Although the loss of toxin by diffusion is negligible the yield in this step is usually very small. Toxin titers of 1:10,000 and 1:100,000 often dropped to 1:1,000 and below. It has been impossible to account for his loss, but further work to elucidate the factors is in progress.

Evaporation.—The salt free toxin solution was evaporated in vacuo at 30 to 50 C. (mostly 40) and dried in a desiccator over concentrated H_2SO_4 and solid NaOH. Experience has shown that a temperature of 50 C. did not injure the purified toxin.

Yield.—Nine different lots of toxins have been prepared. The yield of the dry substance varied considerably in the different experiments; two liters of culture fluid have given from 0.2 to 4.9 Gm.; values of 1 to 2 Gm. are most frequent. These variations may be attributed to the constant enzymatic activity which changes the solution during the different operations, e. g., the longer the dialysis lasts the more protein material is built down and diffuses.

The MLD of the dry preparations range from 0.2 to 4.0 μgm. for a mouse (1 μgm. = 1 microgram = 10^{-6} Gm.). The factors which control the production of a toxin of high and constant potency have not been established. Consequently the total yields expressed in the number of MLD recovered vary between 0.15 and 1.8%. It has already been stated that the greatest loss of toxin occurs most frequently during dialysis. A number of explanations could be considered, but since the experimental proof is not available their discussion is reserved for a future publication.

TABLE 1

Preparation of Dried Toxin (1)

Procedure	Amount, Cc.	Toxic in Dilution of	Total Number, MLD
Toxin (1)	1,500	1:50,000	75,000,000
Adsorbed with 1,500 cc. of aluminum hydroxide			
Filtrate	3,000	1:10	Loss 30,000
Elutions with 0.5% $(NH_4)_2HPO_4$ *			
1	500	1:10,000	5,000,000
2	500	—	—
Combined	1,000	1:2,000	2,000,000
½ of elutions dialyzed	500	1:1,000	500,000
Dried substance	1.825 gm.	4 μ gm.	456,000
Calculated total yield 1.2%	3.650 gm.	4 μ gm.	912,000

* Without addition of glycerol.

TABLE 2

Preparation of Dried Toxin (2)

Procedure	Amount, Cc.	Toxic in Dilution of	Total Number of MLD
Aged toxin (one year) (2)	2,000	1:100,000	200,000,000
Adsorbed with 2 liters of aluminum hydroxide			
Filtrate	4,000	1:1,000 *	Loss 4,000,000
Washwater	200	1:1	200
Elutions with 1% $(NH_4)_2HPO_4$			
1	750	1:100,000	75,000,000
2 (discarded)	750	1:1,000	750,000
Elution 1 dialyzed	750	1:10,000	7,500,000
Evaporated, weighed	0.930 gm.	0.4 μ gm.	2,300,000
Yield 1.1%			

* Incomplete adsorption was probably due to the high nitrogen content of the culture fluid, as 10% gelatine had been added to the usual medium.

Examples of the procedure just outlined are given in tables 1 and 2. The yields in each step are reported as MLD.

Variations of Routine Procedure.—Numerous attempts were made to isolate more potent toxins by modifying the routine procedure. Repeated adsorptions by aluminum hydroxide failed to materially increase the toxicity of the product, although the yields were much better. In one case the MLD was improved from 4 μgm. to 2 μgm. and 66% of the toxin units were recovered. Preliminary adsorption of nontoxic protein material by kaolin (Merck U. S. P. VIII) did not change the properties or the toxicity of the end product. Treatment with

kaolin after the aluminum adsorption caused the titer of the solution to drop and rendered it useless. It is quite likely that the removal of a protective colloid which stabilized the toxin is responsible for the loss. Dialysis of the culture fluids preliminary to the aluminum adsorption offered no advantage. Slightly higher toxin titers were sometimes encountered but the yields of dry substance were considerably smaller.

For comparison one lot of dry toxin was prepared by precipitation with ammonium sulfate. In two liters of toxin 1,500 Gm. of $(NH_4)_2SO_4$ were stirred, the solution warmed to 34 C, and the sticky precipitate skimmed off. It dissolved clearly in water; the adhering salt was dialyzed and the toxin solution evaporated in vacuo. In this manner 1.5 Gm. of dry substance with a toxin titer of 2 μgm. were obtained. The total yield was the same as by the adsorption method and the products did not differ in toxicity or chemical reactions.

Chemical Properties.—The various dried toxins, regardless of the mode of their preparation are alike in their physical and chemical behavior. They are light brown, dry powders which are readily soluble in water. The toxin is precipitated from its solution by heavy metal salts and other protein precipitants. The preparations give most of the color reactions for protein products, but differ from each other in the strength of the different tests. No relation could be found between the intensity of any of the reactions and the toxicity. The comparative tests were made on 0.2% solutions (approximately 1000 MLD); in doubtful cases concentrated solutions were employed. The biuret test although faint is positive in all cases. The Millon reaction is slight; upon boiling the precipitate turns to a light brown, whereas the solution remains colorless. The diazo test on the other hand is invariably pronounced. A few preparations give a very faint Adamkiewiecz reaction. In two products the absence of a free amino acid is evident from the negative reaction of Waser and Brauchli.[10] The Molisch test for carbohydrates is faintly positive with all toxins. From these results it is quite apparent that the toxin preparations are still of a complex nature and variable composition. No attempts have therefore been made to determine their elementary constituents.

As could be expected ferments originally present in the culture fluids were isolated along with the toxin. Several dried products were investigated for their proteolytic action. They were found to liquify gelatin and increase the amino nitrogen of Witte's peptone. Both properties vary with the different preparations and are not in quantitative relation to the toxin titers. Urease was not found. The enzymatic properties of the toxic product will be discussed more in detail later.

[10] Helvetica Chim. Acta, 1924, 7, p. 740.

SUMMARY

Isolation of botulinum toxin has been attempted by selective adsorption on colloidal aluminum hydroxide, elution with secondary ammonium phosphate, dialysis and evaporation at 40 C. Dried toxins with titers of 0.2 to 4.0 μgm. per MLD for the mouse have been obtained. All preparations give slight protein and ferment reactions. Precipitation with ammonium sulfate has yielded a toxin with similar properties.

A COMPARATIVELY SIMPLE TECHNIC FOR THE BACTERIOLOGIC STUDY OF FECAL MATERIAL

FOR CLINICAL AND EXPERIMENTAL PURPOSES

JOHN C. TORREY

From the Department of Hygiene, Cornell University Medical College, New York

The development of effective methods for the bacterial examination of intestinal material during the past few decades has led coincidentally to greater knowledge of the types of bacteria found in the intestine of man and lower animals. The organisms causing disease have naturally attracted particular interest, and for the detection of such specific pathogens as B. typhosus, B. dysenteriae and V. cholerae, there are now available excellent selective mediums and methods. Methods, suitable for more general studies of intestinal floras, however, have been on the whole either unnecessarily complicated and time consuming, or inadequate for a comprehensive survey of the various bacterial types in the material.

Herter and Kendall[1] were among the first to suggest a combination of culturable tests applicable for general study. They stressed the value of the fermentation of different sugars in nutrient broth cultures of the whole stool specimen, believing that the degree of gas production and the microscopic examination of gram-stained preparations from the bacterial sediment revealed in a general way, the abnormalities of the flora. Many of their mediums and methods have been used, particularly by investigators in this country (MacNeal, Latzer and Kerr,[2] Torrey,[3] Rettger and his coworkers,[4] Morris, Porter and Myer,[5] Cannon,[6] Goldman[7] and others), and additional helpful procedures suggested.

For a number of years past I have been working for a bacteriological technic which would be simple enough to be practical for general use and yet adequate for a comprehensive analysis, both qualitative and quantitative, of the intestinal flora in health and disease, including the search for the causative bacterial agents in acute and chronic colitis and

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1 J. Biol. Chem., 1909, 7, p. 203.
2 J. Infect. Dis., 1909, 6, pp. 123, 571.
3 J. Infect. Dis., 1915, 16, p. 72; J. M. Research, 1919, 39, p. 415.
4 Rettger and Cheplin: The Intestinal Flora, 1921. Hull and Rettger: J. Bact., 1917, 2, p. 47.
5 J. Infect. Dis., 1919, 25, p. 349.
6 J. Infect. Dis., 1921, 29, p. 369.
7 J. Infect. Dis., 1924, 34, p. 459.

the study of the possible relation of intestinal bacterial conditions to various systemic or local diseased conditions of obscure etiology, such as certain types of anemia, nervous disorders, chronic arthritis and general debility. The value of the technic adheres to the fact that each of the three mediums, when employed as directed, serve a definite and useful purpose and that in combination they so complement each other as to provide a basis for as complete a bacterial survey as may be desired.

A detailed description of the various technical procedures will be given first, leaving to a subsequent section a brief discussion of their respective significance.

PRELIMINARY PROCEDURE

Clinical Material.—This should be examined as soon as possible after collection, certainly within 2 or 3 hours. The manner of obtaining the material is dependent on the purpose in view. If a bacterial analysis of the rectal region is desired a normal movement may be examined or the material obtained through the aid of a proctoscope, with or without a preliminary catharsis and irrigation, but if that from higher colonic levels is required, some such cathartic agent as epsom salts, phenolphthalein or citrate of magnesia should be given and the specimen selected from the second or third movements, or better still from the result of a high irrigation with sterile water. Particularly, in cases of colitis it is important to obtain some mucus. This should be well washed in sterile salt solution and part spread on a slide for staining and microscopic examination and part streaked on the purple agar plate medium described below. If an examination of material from intestinal segments removed at operation is to be made, some of the lumen content is collected and with it scrapings from the gut wall. This should be placed in test tubes with rubber stopper and packed in ice prior to examination.

Reaction.—The reaction of the material may readily be ascertained by the use of weak, aqueous (neutral) dilutions of the Clark and Lubs indicators, brom-cresol purple, brom-thymol blue or phenol red, according as the reaction is acid, nearly neutral or alkaline. A little of the material, about the size of a pea, is picked up on a glass rod or inoculation loop and rubbed into suspension in a tube containing about 10 cc. of the proper indicator and, after standing for a few minutes to allow for settling or after centrifuging, the color obtained is compared with known H-ion standards. This test should be made as soon as possible after the collection of the specimen.

Preparation of Fecal Suspensions.—In order that an examination may have comparative value as regards quantitative bacterial findings, it is necessary to adopt some standard for the degree of density of the primary fecal suspension. The use of a nephelometer of the McFarland type, as recommended by Rettger and Cheplin,[4] has its advantages but as this or a similar instrument is not generally available, the following simple procedure is recommended and is believed to be sufficiently accurate for the purpose in view:

A small amount of the well mixed fecal specimen taken up on a glass rod with a small prong at the end, is evenly suspended in about 20 cc. of salt solution in a test tube with an inside diameter of 16 to 17 mm. This is allowed to stand

for several minutes to permit the coarser particles to settle and is decanted into another similar tube. A heavy mark with a wax pencil (black preferable) is then made about one half way up on the outside wall of the tube and the density of the suspension adjusted either by rubbing up more material or by adding salt solution until the mark is just discernible as a faint shadow in looking through the tube toward a strong light. This is labeled tube 1 and from it dilutions are prepared in a series of 8 tubes, each containg 9 cc. of salt solution and 1 cc. of the preceding dilution. Tube 9 contains, accordingly, a 1 : 100,000,000 dilution of the primary suspension in tube 1. If the latter has been properly prepared, tube 3 will show only a faint cloud, just discernible when viewed in a good light against a dark surface. In the examination of material from the small intestine, the primary suspension is generally necessarily much lighter—about equal to the density of tube 2 of the above series.

Five cc. are next removed from each of the dilutions, 1, 3, 5, 7 and 9 and are placed in narrow (10 mm. inside diameter) tubes. These are heated in a water bath at 80 C for 10 minutes, thereby killing all but the strongly heat resistant organisms (chiefly spore-bearers, although some streptococcal and B. aerogenes types may survive).

Microscopic Examination.—Except for special purposes, such as the examination of infant stools, of material from different segments of the intestine, and following the degree of implantation of B. acidophilus, gram-stained films of fecal material generally give little or no definite information and may be omitted. If carried out, one of the alkaline modifications of the original gram-method, such as that of Kopeloff and Beerman,[8] is recommended. This technic gives a much sharper differentiation than the original method. Mucus spread thinly on slides and air dried, may be stained with this method, with carbol-thionin (Lyon-Smith[9]) or with a tubercle bacillus stain, if such a procedure seems called for.

CULTURAL PROCEDURES

From a large variety of tested mediums three have been selected as providing in combination an adequate basis for a comprehensive bacterial analysis of fecal material whether for specific or general purposes: Brom-cresol purple lactose agar plates, surface seeded, as a differential medium for the aerobic types; glucose blood agar plates, surface seeded, and incubated under reduced oxygen tension; and cooked meat medium in tubes for the anaerobic types and to serve as a presumptive differential test for the spore bearing anerobes and to indicate the putrefactive propensities of the whole flora.

*Brom-cresol Purple Lactose Agar.**—This medium is prepared as follows:

500 gm. of chopped beef heart (other meat material may be used) are boiled in 1 liter of water for 15 minutes over the free flame. Strain through canton flannel and absorbent cotton as usual. Make up loss in filtrate with water and add 10 gm. peptone and 15 gm. flaked agar. Heat in Arnold until in solution. Adjust reaction to PH 7.4, and place in Arnold for 30

[8] Proc. Soc. Exper. Biol. and Med., 1922, 20, p. 71.

[9] Lancet, 1920, 1, p. 1268.

* The dehydrated Difco medium may be used but the reaction should be adjusted to PH 7.4. The medium prepared from fresh meat, however, gives development to a greater variety of bacterial types and has better differential value.

minutes. Readjust reaction, if necessary. Filter through absorbent cotton and add to filtrate 10 gm. lactose, and sufficient saturated alcoholic solution of brom-cresol purple (generally about 0.75 cc.) to give the desired depth of color for a poured plate. Put in flask in 200 cc. amounts (sufficient for 6 plates) and autoclave at 15 lbs. for 10 minutes.

Large petri dishes (120 mm. in diameter) are used and plates prepared with the above medium. At least three are desirable for an examination and after the agar has cooled and hardened they are surface-seeded respectively with a large loop (0.4 cm. in diameter) of fecal suspensions in tubes 4, 3 and 2. The loop of material should be drawn across one side of the plate and then distributed thoroughly over the whole surface of the medium. If after seeding there is any evidence of moisture on the medium surface it should be dried off by partial uncovering and exposing the plate face downward in the incubator for a few minutes.

These plates are incubated for 24 to 48 hours at 37 C and then kept at room temperature for 2 to 3 days longer for further study. Staphylococcus aureus colonies are particularly conspicuous after this period. They bring to development practically all of the aerobic types in the flora and nothing is to be gained by enrichment with ascitic fluid or a similar agent. This medium has marked differential value and if seeded in accordance with the above directions these plates give quantitative information as well. The B. coli-aerogenes group develop as yellowish colonies with a yellow zone. B. mucosus forms mucoid colonies with color unchanged or slightly yellowish; B. typhosus and B. dysenteriae colonies, thin, translucent, smoky hue, with medium unchanged; B. paratyphosus (intermediate) group like B. typhosus or medium darker purple with blue centers in colonies (B. morgani, B. alkaligenes); B. proteus, irregular spreading contours, dark reddish purple zone; B. pyocyaneus group, yellow colonies with greenish purple zone. For a general analysis of the flora for specific purposes representative colonies may conveniently be fished and seeded on Russell double sugar slants and then carried through differential mediums. A great variety of coccus types develop on these plates. These may be differentiated by fishing representative type colonies and streaking on plain blood agar plates (P_H 7.4). The effect on the blood agar, chromogenesis morphology and reaction to the gram stain, seeding into differential sugars (lactose, sucrose, salicin, raffinose and mannitol) and into litmus milk and gelatin stab will generally serve for identification.

Virulence tests for B. coli or B. paratyphosus-like types may be made by seeding into meat-infusion peptone broth (P_H 7.0) and incubating for 24 hours. Dilutions of the cultures are made in sterile salt solution to 1 : 15 and 1 : 50. Two white mice (20 to 25 Gm.) are then inoculated intraperitoneally, one with 0.5 cc. of the 1 : 15 and the other with 0.5 cc. of the 1 : 50 dilution. As regards the colon types, if the strain is avirulent neither will die, if moderately virulent, the first but not the second will die within 48 hours, and if exceptionally so, both mice will die within that period. After inoculation with paratyphoid-like types the mice should be observed for 4 to 5 days to cover a more prolonged incubation period.

Reduced Oxygen Tension Plates.—The medium for these plates is nutrient glucose agar (meat or liver infusion, 1% peptone, 1.5% agar, 0.5% glucose, P_H 7.4) to which 10%, fresh defibrinated sterile blood has been added just before the plates are poured. Deep petri dishes (20 mm.) should preferably be used. Oxygen tension reduction is obtained by B. subtilis growth on an opposed agar plate in accordance with the technic recently described by Herrold[10] for gono-

[10] J. Am. M. A., 1921, 76, p. 225.

coccus culture. This was in turn a modification of a method described[11] some years ago in which the Wherry and Oliver[12] technic was adapted to plate cultures.

Two sterile bottoms of deep petri dishes are selected which are exactly of a size. Into one the blood agar medium is poured and when hard is surface-seeded with diluted fecal suspension, drying off, by inverting in the incubator, any obvious surface moisture after seeding. Into the other dish about 10 cc. of a firm nutrient agar, seeded with B. subtilis (a type producing a thick growtb, such as B. cereus, is preferable) is poured. When hard it should be dried off in the incubator until no moisture is visible. These two bottoms are then fitted together and bound with 1 incb width adhesive tape, and this in turn is covered with broad elastic bands.

One blood plate should be surface-seeded with a large loop (4 cm. diameter) from dilution tube 4, and one from dilution tube 3. They should be incubated for at least 48 hours with the blood agar side uppermost. This method will at times bring to development a surprisingly large variety of organisms, some of which have not been recovered in any other way. B. bifidus and B. acidophilus, grow particularly well and may be recognized by the brown or chocolate color and morphology of the colonies. At times the colony count for these plates is many times that for aerobic plates seeded from the same fecal dilution and on the same medium.

Cooked Meat Medium.—This medium is prepared according to the original description of Robertson,[13] except that peptone is added and the reaction adjusted to P_H 7.2, as follows:

Beefbeart (fresh and finely minced)	500 gm.
Peptone	10 gm.
Water	1 liter

This mixture, with the meat particles evenly suspended, is cooked in a double boiler with just enough beat to cause a slight simmering for about 10 minutes and the reaction adjusted to P_H 7.2. The cooking is then continued for about 1½ hours and tbe reaction readjusted, if necessary. The broth is decanted and the meat is then placed in test tubes, 16 to 17 mm. inside diameter, so that there is a column about 5 cm. high. After autoclaving these tubes and the broth in the flask at 15 lbs for 15 minutes, the latter is added to the meat tubes in 5 c c. amounts. The tubes are tben placed in the Arnold on two successive days for 20 minutes. This method prevents the fluid being forced up into the cotton plug as is apt to occur if fluid and meat are placed together in tubes and then autoclaved.

Just before seeding, the seven tubes necessary for the examination are placed in the Arnold for 15 minutes and are then cooled to 45 C. One tube is next seeded with 1 cc. of unheated dilution 5 of the specimen, placing the tip of the pipette rather deep in the meat, and one tube with 1 cc. of the unheated suspension 1. Five other tubes are seeded respectively with 1 cc. from dilutions 9, 7, 5, 3 and 1, heated at 80 C. The height of the meat column in each tube is then registered by a wax pencil mark on the glass and all are capped by running about 5 cc. of melted, warm, sterile yellow vaseline on the surface of the fluid in each tube. They are then incubated at 37 C. and are examined after 1, 3 and 6 or 7 days. At each observation the degree of digestion is measured in mm. as shown by the decrease in the meat column height, the amount of gas noted (elevation of the vaseline cap) and also changes which may have occurred in the color and composition of the meat as indicated in the table. The tube seeded with the unheated fecal suspension 1 yields information particularly in reference to the putrefactive propensities of the whole flora. If the decrease in the meat column height amounts within a week to 1.5 to 2.0 cm. an unusually marked putrefactive propensity is indicated; if between 1.0 and 1.5 cm. a fairly marked; and less than 1.0 cm., a moderate to slight tendency. The digestion is very largely due to the spore bearing anaerobes.

[11] J. Bact., 1917, 2, p. 435.
[12] J. Bact., 1916, 19, p. 288.
[13] J. Path. & Bact., 1916, 20, p. 327.

As is illustrated in table 1, this medium seeded with the heated fecal dilutions as directed, serves as a presumptive differential test for the presence of most of the intestinal spore-bearing anaerobes, and permits, in some degree, a quantitative estimation. Where desirable, these anaerobes by appropriate method (Kahn [14]) may be isolated and identified, the predominant type being often found in pure culture in one of the tubes seeded with the higher dilutions of the heated fecal suspension.

DISCUSSION

Reaction.—The reactions of stool specimens from normal adults under conditions of ordinary diet varies greatly. Although, the majority of such specimens have shown reactions between P_H 6.6 and 7.0, not infrequently more acid and also slightly more alkaline samples may be encountered. Rettger and Cheplin [4] have reported reactions between P_H 6.0 and 6.4 in normal adult individuals.

Among pathological conditions in which acid reactions are frequent, are cases of acute eczema and allied skin diseases, of excessive fermentation with flatulence, and particularly of chronic ulcerative nonspecific colitis. In the latter group, stool specimens generally show reactions between P_H 5.5 and 6.2. Reactions more alkaline than P_H 7.2 are much less frequently observed. In the majority of such cases, the flora is markedly putrefactive and there is often a condition of intestinal stasis. Reactions more acid than 6.0 and more alkaline than 7.2 are indicative of pathologic conditions, or of abnormal bacterial conditions as regards types or activity. Although no very definite correlation could be established between the reaction of stool specimens and types and numbers of bacteria, in general it may be said that the more acid specimens showed a predominance of actively fermenting strains of the colon group. B. welchii also might be particularly in evidence, but, on the other hand, this organism in unusual numbers is at times encountered in definitely alkaline specimens. In such latter specimens, also, putrefactive spore bearing bacteria such as B. sporogenes, B. putrificus and B. bifermentans, and also peptolytic nonspore-bearers of the proteus or alkaligenes type are likely to be found. More rarely, the alkalinity appears to be principally due to representatives of the colon group with strong aminolytic tendencies. The reaction of fecal specimens bears no relation whatever to the acidity in the gastric secretion but seems to be determined largely by the trend of bacterial activities in the large intestine, particularly, by that of the colon group.

[14] J. M. Research, 1922, 43, p. 155.

Brom-Cresol Purple Lactose Agar Plates.—This medium is recommended for the present purposes in preference to Endo medium. It is less troublesome to prepare, more likely to be uniform and brings to development a greater variety of aerobic organisms. It is far superior for streptococcal types, and differentiation between them is generally more distinctive and clear cut. As has been stated, however, Endo medium is preferable for differentiation in the colon-paratyphoid group. There is nothing to be gained by adding any enrichment agent such as ascitic fluid to the medium.

Morris, Porter and Meyer [5] in the course of their study of the fecal flora of infants, found that even a casual inspection of surface seeded Endo plates supplied much valuable information in regard to the character of the flora, plates seeded from putrefactive stools showed a heavy growth formed by a considerable variety of organisms and the medium became strongly alkaline with a pungent musty odor after 48 hours or more incubation; whereas with a fermentative flora the colonies are generally fewer, mostly B. coli and streptococcus types, and the medium reaction remains acid with a marked acid odor. These differences are evident on these purple lactose agar plates and, accordingly, they should be observed for four to five days for changes in reaction. This is particularly true for stool examinations of infants and young children where the differences in reaction induced by these two types of flora are perhaps rather more distinctive than with adult fecal specimens. In some specimens from the latter group, however, in which the flora has a marked putrefactive tendency, these plates change from an acid to a marked alkaline reaction within the above period as shown by the development of a general deep purplish blue color.

Aside from this indication of the general qualitative character of the flora, the plates permit a quantitative estimate of aerobic bacterial types, particularly, of the colon and streptococcal groups.

MacNeal, Latzer and Kerr [2] in repeated examinations of fecal specimens from 12 normal adults, found that the number of colonies on their litmus lactose agar plates (aerobic) ranged from 1,360 to 1,500,000 per mg. of fecal material, with the highest average for one individual of 280,000 and the lowest in another of 33,000. These figures were taken to represent approximately the number of cultivatable B. coli. My observations have indicated that a count of about 100 B. coli colonies on fecal dilution 4 brom-cresol purple lactose agar plate, surface seeded as described above, signifies approximately a viable B. coli content of 300,000 per mg. of fecal material with the average moisture content. This estimate has been reached by comparing in a considerable number of examinations, the B. coli

colony count on these plates with that of Ayer and Rupp[15] agar medium (selective for B. coli) poured plates seeded with 1 cc. of the same fecal dilution. It is unusual to find regularly a count as high as that for fecal specimens from individuals with normal digestion and elimination and under ordinary dietary conditions. Under these circumstances the dilution 4 plate is likely to show a colony count for B. coli ranging from about 5 to 50. In a small percentage of seemingly entirely normal individuals, however, the intestinal flora is characterized by an exceedingly small count of B. coli, and this will be found to be the case in repeated examinations; in fact, in one individual a colon bacillus was found only with difficulty. In view of the recent reports by Hosoya and Kuroya[16] on the water-soluble vitamin which B. coli seems to be able to synthesize, it would seem possible that the presence of a normal number of these organisms in the intestine might have an influence of some importance in nutrition, and the prevention of a vitamin deficiency condition, particularly, if the individual's diet is constantly deficient in this element. Ordinarily, however, the diet of most individuals contains an abundance of water-soluble vitamin and the presence or absence of that element elaborated by B. coli in the intestine would seem likely to be of slight significance.

Among the pathologic conditions in which unusually high counts of B. coli may be encountered the more outstanding are colitis, particularly the ulcerative type and certain constipative conditions with marked flatulence. There is a general impression that stool specimens from cases of chronic constipation are characterized by a marked paucity of viable bacteria. It has been the writer's experience, however, that a high count is more often found unless pronounced stasis with inspissation of the fecal material has occurred. On the other hand unusually high counts of B. coli in fecal specimens may be entirely compatible with normal conditions and functioning of the intestine: certain colon strains tending, as is well known, to inhibit putrefactive bacteria, and, perhaps, through their fermentative products stimulating peristalsis.

In investigating the possible relationship of certain B. coli strains to more or less chronic intestinal disorders, such as colitis, much more weight should, of course, be given to degree of virulence than to mere numbers.

An unusual degree of virulence is indicated by the fact that 0.02 to 0.01 cc. of a plain broth B. coli culture injected intraperitoneally will kill a 20 to 25 Gm. white mouse within 24 to 48 hours; whereas, with an ordinary nonvirulent colon strain, a dosage of 0.05 cc. or more is well tolerated. These mice show at necropsy a marked hyperemia of the subcutaneous, intestinal and pleural blood vessels with small areas of extravasation into the surrounding tissues, and with some strains, petechial hemorrhages into the small intestine, pleural cavity and urinary bladder. Most of the virulent strains also cause a marked hyperplasia of the lymphoid tissues, especially, the axillary and inguinal lymph nodes, the solitary follicles of the small intestine and the spleen. The production of these lesions

[15] J. Bact., 1918, 3, p. 433.
[16] Scientific Reports from Gov. Inst. for Infect. Dis. (Tokyo), 1923, 2, p. 287.

seems dependent on the ability of the particular colon strain to multiply within the body, and possibly the blood stream, of the animal as cultures from the heart at the time of death yield very high colony counts.

These more virulent B. coli strains have been recovered in chronic intestinal disorders, not alone of a colitis type but also from cases of auto-intoxication in which the conditions seem clearly related to the intestine and also from individuals who are subject to occasional intestinal disturbances. Several hundred B. coli strains have been studied from the virulence standpoint and the findings will be reported in detail in another article. It may be said, however, that these virulent strains are by no means common and that their presence in any case would seem to be of definite significance.

An inspection of these purple agar plates also yields information as regards the ratio to B. coli, of streptococci, staphylococci, B. mucosus, B. lactis aerogenes, and the alkali producing organisms.

This latter group includes B. proteus, B. pyocyaneus and related green pigment producing bacilli, and certain members of the "intermediate" (paracolon) group. These alkali producers are found in more than insignificant numbers only in markedly proteolytic floras. It is very unusual to find B. proteus in human feces, and its constant presence is indicative of an abnormal intestinal condition which favors putrefaction. B. acidophilus, if present in more than the usual negligible numbers, may be found after 3 or 4 days' incubation, forming small, scale-like colonies in close proximity to the B. coli colonies or growing on their surface as papillae. Rarely these papillae have been found to be formed by B. bifidus. To follow satisfactorily, however, the attempted implantation of members of the lactobacillus group or their response to dietary conditions, it is necessary to employ suitable mediums such as the whey agar of Rettger and Cheplin[4] or the liver glucose agar.[11]

There has been a great deal written about the types of streptococci in the human intestine but no simple method has been found as yet to differentiate the pathogenic from the saprophytic strains. It is hardly necessary to state, however, that the mere presence of very large numbers of the type commonly found in the large intestine (Micrococcus ovalis, Enterococcus or Streptococcus fecalis as it is variously called) means little or nothing in itself. The relative numbers of this type depends very largely on the diet. In adults, particularly, on a low protein, high carbohydrate diet, they are likely to become the dominant type in the flora, in fact, much more likely than is B. coli (also observed by N. and J. Mutch,[16a] or B. acidophilus. These enterococci differ considerably in cultural features. They may or may not be glycophilic, acid and heat resistant. They are generally described as mannitol

[16a] See later reference.[26]

fermenters but it has been my experience that strains from individuals suffering from one type or another of intestinal derangement (constipation with flatulence, colitis, etc.) are quite as likely to be negative as positive as far as the splitting of mannitol is concerned. In 44 such cases mannitol nonfermenting types were found in 24 instances and mannitol fermenters in 20, with occasionally a mixture of the two.

It has frequently been claimed that certain of these enterococcus types may at times be pathogenic and the etiological factor in colitis.

Bargen,[17] however, seems to be the first to submit definite experimental evidence that such may be the case. With pure broth cultures of some strains of these streptococcal types recovered from the ulcers of chronic colitis cases, he was able to cause a violent diarrhea in rabbits, often with blood and mucus in the stools, after intravenous inoculation. In these animals at necropsy, lesions were found in the rectum and upward, consisting of "disseminated petechial hemorrhages and massive subcutaneous hemorrhages to superficial ulcers of the mucosa." The lesions, however, were generally more extensive and diarrhea more severe in animals receiving mixed cultures of the gram-positive diplococcus and the gram-negative bacilli (presumably B. coli), which would suggest that both these types played a part in producing the colitis condition from which they were isolated. Bargen describes these gram-positive diplococci as "plump with a tendency to lancet-shaped—about the size of pneumococci—no capsules, little tendency to grow in chains and on blood agar plates forming colonies resembling the alpha hemolytic streptococcus (Brown)." Sugar tests, made on a few strains, gave negative results with mannitol and inulin and hence were not Streptococcus fecalis. His description and illustration suggest that they conformed to one of the mannitol nonfermenting variants (Dible[18]) of the enterococcus group. Drew,[19] however, described enterococci isolated from washed mucus of colitis cases as mannitol fermenters and difficult to cultivate, anaerobic conditions being necessary at first. Although enterococci may be a factor in the etiology of ulcerative colitis, it would seem quite impossible to differentiate the pathogenic strains by cultural tests alone.

Obviously, the most conclusive criterion of pathogenicity are positive results following animal inoculation but as is well known for the streptococcus group, and as Bargen found for his strains, such tests are frequently unsuccessful. Unless streptococcal strains of this type are found in predominating numbers in well washed mucus obtained during acute attacks of colitis or from the ulcers in the chronic type, little significance may be attached to their presence in the intestines of such cases.

Occasionally, I have recovered from fecal specimens a gram-positive diplococcus in colony formation, morphology, and sugar fermentations very similar to Streptococcus fecalis, but which differs from it in being markedly proteolytic.

[17] Collected Papers of the Mayo Clinic, 1924, 16, p. 24.
[18] J. Path. & Bact., 1924, 24, p. 3.
[19] J. State Med., 1918, 26, p. 217.

Most strains have been found to split glucose, lactose, sucrose, maltose, salicin and mannitol whereas a few fail to ferment sucrose. In milk, an acid clot is formed in 24 hours' incubation but this shows evidence of digestion within 1 to 3 days which progresses until within a week, the curd is reduced to ½ to ⅔ its former size. A tube of gelatin is liquefied completely within 7 to 14 days and solidified serum (Loeffler slants) slightly within that period. This type has been recovered from 8 of 132 persons with abnormal intestinal conditions, and in repeated examinations where this was done. Five had colitis of the chronic type, and three had constipation with toxic symptoms. It has not been found in specimens from normal persons and when present at all in pathologic conditions it is always found in large numbers. This type is in every respect identical with M. zymogenes of McCallum and Hastings,[20] which they recovered from the blood of a case of endocarditis and which has subsequently been recognized as occasionally the etiologic factor in that condition.

Whether or not it acts pathogenically in the intestine is not known but I believe it may multiply actively at as high levels as the cecum, and that possibly the intestine may serve as the focus of infection in the rare instances when the blood stream is invaded. At all events its presence in the large bowel indicates putrefactive conditions. As far as I am aware this is the first time this organism has been definitely identified as an intestinal habitant.

Another of the rarer intestinal streptococcal types is the Streptococcus salivarius.

According to the Holman[21] classification Streptococcus salivarius is nonhemolytic and ferments lactose and sucrose but not salicin or mannitol. Of the 323 streptococcal strains from normal individuals studied by Oppenheim,[22] it constituted only 1%; Golman[23] found it only once in her study of the flora of the small intestine of pathological subjects and in that instance in the jejunum. In my series of 132 individuals, nearly all adults, I found it 8 times and in 5 of these the stool specimens were from cases of colitis. On rabbit blood agar (P_H 7.4) it produces colonies with a narrow greenish zone of partial hemolysis in 24 to 48 hours and appears as a small, nearly round, gram-positive coccus in diplococcus and short chain form. This type has been frequently encountered in focal infections of the nose and throat but evidently does not ordinarily thrive in the intestine. Its occasional relationship to inflammation of the intestinal mucosa has not been definitely established but when recovered in considerable numbers and in repeated examinations of washed mucus from a colitis case, the finding would seem of significance. Rarely such a case is encountered and an autogenous vaccine prepared with the salivarius strain is worthy of trial.

There is general agreement among those who have investigated the matter, that the true hemolytic streptococcus (beta type) is rarely encountered in the human intestine.

[20] J. Exper. Med., 1899, 4, p. 521.
[21] J. M. Research, 1916, 34, p. 377.
[22] J. Infect. Dis., 1920, 26, p. 117.
[23] Ibid., 1924, 34, p. 459.

Oppenheim[22] recovered only 5 strains from 15 normal persons whose stool specimens were repeatedly examined. Dible[18] found only two hemolytic strains among 152 recovered from feces. Wordley[24] using Dudgeon's method of drying out fecal material on sterile porus tiles recovered comparatively few hemolytic streptococci. Of 52 streptococcus strains studied, 13 were hemolytic on blood agar and of these only 4 were the long chained variety, 7 were diplococci, 4 fermented mannitol and 7 did not. In a later study of 76 cases of scarlet fever in the first week, however, he[25] recovered hemolytic types from the feces of 24, and also from 16 of 33 cases of acute nephritis. Goldman[23] in an examination of material from the jejunal region of 4 normal and 15 pathological cases, did not encounter any hemolytic streptococci nor did N. and J. Mutch[26] in a similar study. Farr,[27] also, failed to recover any hemolytic streptococcal strains in a series of 131 chronic and acute appendices. The above citations are representative of numerous reports in the literature and are mainly in line with my findings. Furthermore, when hemolytic streptococci have been found in cultures from intestinal material, they have generally been reported as few in number. Under such conditions they may be considered of very questionable significance as they might well have been derived from the throat or from food (milk).

It has been the experience of the writer that the hemolytic type is rarely associated with ulcerative colitis. In only one case in a series of over 20 has this type been recovered from stool specimens from such cases. In this patient, examined through the courtesy of Dr. Jerome M. Lynch, a beta type of hemolytic streptococcus was found in very large numbers in both the washed mucus and fecal material and in two examinations about 6 weeks apart.

This strain formed on plain rabbit blood agar scale-like, dry, fragile colonies about 2 mm. in diameter after 1 day incubation with a zone of complete hemolysis 1.5 mm. wide. In ½% hormone agar it formed chains varying in length and made up of gram-positive, small round cocci which varied in size and collected in dense tangles. In sugar fermentations it conformed to Streptococcus angiosus (Holman).

This patient showed punched out ulcers in the sigmoidal and rectal region with mucopurulent matter overlying the mucosa. In a personal communication Dr. L. Mary Moench has informed me that she made a similar finding at the Mayo Clinic in connection with the study of a case of ulcerative colitis in which the infection involved the abdominal wall. On repeated examinations a long chained hemolytic streptococcus was recovered in large numbers and this strain was also proved to be pathogenic. Cases of this type, however, are extremely rare and all the evidence points to the conclusion that the beta (hemolytic) type does not thrive in the human large intestine and is very unlikely to infect its wall.

Another streptococcal group, to which little attention has been given, as far as their occurrence in the intestine is concerned, are those which require anaerobic conditions, either partial or complete. During the cultural examination of bits of rectal mucous membrane from a small series of cases of ulcerative colitis at the St. Bartholomew's Hospital and through the cooperation of Dr. Lynch, I isolated a number of these strains. At first, all grew only under conditions of reduced oxygen

[24] J. Hygiene, 1921, 20, p. 60.
[25] Lancet, 1922, 2, p. 610.
[26] New York M. J., 1921, 113, p. 713.
[27] Ann. Surg., 1921, 73, p. 749.

tension and some continued to required these conditions whereas others later thrived under aerobic conditions. Cultural studies carried out by Dr. M. C. Kahn in this laboratory indicated that these strains varied as greatly as regards morphology and fermentations as do the aerobic types. They developed in semi-solid hormone agar, for the most part in short chains of cocci, irregular in shape and size, some being round, others compressed and others bacilloid. Tested in lactose, salicin, raffinose and mannitol broth medium, it was found that some strains fermented none of these carbohydrates, others all of them and still others all except mannitol. Our finding indicated that these anaerobic types thrived best on or buried in the intestinal mucous membrane. The pathogenicity of some strains of anaerobic streptococci has been well established, and it is possible that the intestine may serve as a point of invasion at times. Two of these intestinal strains inoculated into rabbits and guinea-pigs gave negative results. They had, however, been under cultivation for some time when tested.

Blood Agar Plate.—The reduced oxygen tension blood agar plate (subtilis method) is not essential for ordinary examinations but serves a useful purpose in research work on the little known group of intestinal organisms which will grow only in a moderately depleted oxygen environment.

B. bifidus grows readily under these conditions and forms characteristic colonies which have been described in a previous article.[11] There is also a group of nonspore-bearing bacteria with the morphology of slender rods, either gram-negative or gram-positive, which are found in large numbers at times throughout the large intestine and which form colorless translucent colonies on these plates. A detailed discussion of the nature and significance of these types must await further study.

This plate method is also well adapted for bringing to development certain enterococcus-like types, present in mucus which, as Drew[19] noted, favor a preliminary partial anaerobiosis, although the conditions are not suitable to encourage the growth of the strictly anaerobic streptococcal types. Fortunately, also, B. coli colonies grow more slowly than on aerobic plates and thus do not crowd out other bacterial types to the same degree, although spreading types, such as B. proteus, may give trouble. Colonies from these plates may conveniently be fished into 0.5% hormone agar and grown as deep stab cultures.

Presumptive Test for Spore Bearing Anaerobes.—Cooked meat medium, prepared and used as described above, has proved useful as an aid in the study of intestinal material as regards its spore-bearing anaerobe content. The differential value of this medium for this group, which was claimed by its originator (Robertson[13]) has been amply confirmed by numerous investigators.

For the present purposes, it appears to be preferable to minced brain medium (Hall[28]), because of its superior differential value. It not only yields information through characteristic changes in the color of the meat, the degree of digestion and gas production, but also, as Holman[29] has pointed out, because of its buffer action, it permits the simultaneous development of acid producing fermentative types and of proteolytic organisms. It is thus peculiarly well suited for the simultaneous growth of types requiring such diverse cultural conditions as do those frequently embodied in an intestinal flora. Not alone is the degree of putrefactive propensity of the flora as a whole indicated by the digestion and consequent lowering of the meat column, particularly in the tubes seeded with the unheated fecal dilutions, but an inspection of the tubes seeded with the heated fecal dilutions permits, after some experience, at least a presumptive report on the predominating type or types of the more noteworthy spore-bearing anaerobes present in the specimen. This applies particularly to B. welchii, B. bifermentans, B. sporogenes, B. histolyticus and occasionally to Vibrion septique (or B. oedematiens). Partial confirmation may be acquired by examination of gram-stained films and full identification by plating out and differential tests. This last, however, is not necessary for the recognition of B. welchii, B. bifermentans and B. histolyticus. Certain members of this group produce too slight and indefinite changes (B. tertius) or act too slowly (B. putrificus) to give indication of their presence within the test period. Kahn, in studies carried out in this laboratory, made extensive use of the medium in studying both single-cell purified stock strains[14] of this group and of strains recovered from intestinal material.[30] Reference is made to his publications for details as regards the group cultural characteristics, and the frequency with which the various types are found in fecal specimens in health and disease.

It should be stated in passing that the success of this technic largely depends on the presence of the spore stage of these respective types and their concentration. Accordingly, the method cannot be applied so successfully to the study of these anaerobes in the small intestine where they are generally relatively few in number and mostly in the vegetative stage.

Table 1 gives details of the differential changes, which are more or less specific, produced in the medium of intestinal spore bearing anaerobes, by purified strains singly and also in various combinations. The figures designating the degree of digestion are the averages of three tests performed with different lots of medium.

If the medium is to serve as a presumptive indicator of the presence of spore-bearing anaerobes in intestinal material, the reaction must be within the limits of P_H 7.2 to 7.4. As has been stated, presumptive differentiation is indicated by the rate and degree of gas production, digestion of the substrate, and changes in its color and constitution within the specified time intervals. On the acid side of P_H 7.2 gas production is enhanced with all types and digestion decreased, whereas on the alkaline side these tendencies are reversed. If, however, the above reaction is closely adhered to in each lot of medium, the above table will prove useful as a guide.

[28] J. Infect. Dis., 1920, 27, p. 579.
[29] J. Bact., 1919, 4, p. 282.
[30] Kahn: J. Infect. Dis., 1924, 35, p. 423. Moench, Kahn and Torrey: J. Infect. Dis., 1925, 37, p. 161.

TABLE 1

CHANGES IN COOKED MEAT MEDIUM (PH 7.2-7.4) PRODUCED BY INTESTINAL SPORE-BEARING ANAEROBES, SINGLY AND IN COMBINATION

Medium seeded from 24 to 48 hours growths in casein digest broth, 3 to 4 drops.

	1 Day			3 Days		7 Days		
Organisms	Gas*	Digestion, Mm. Decrease of Meat	Color of Meat	Gas*	Digestion, Mm. Decrease of Meat	Gas*	Digestion, Mm. Decrease of Meat	Color of Meat
1. B. welchii	++++ meat blown	0.0	Slight pinkish	++++	0.0	++++	0.4	Copper red, fluid clouded
2. Vibrion septique or B. oedematiens	++ meat blown	0.0	Slight pinkish	+++	0.0	++++	0.1	Pink, fluid clear
3. B. aerofoetidus	+	0.0	No change	Trace	0.2	+	0.3	Light reddish brown
4. B. bifermentans	+	0.6	Reddish brown	+++	0.8	++++	1.0	Dark reddish brown
5. B. histolyticus	0	0.5	Dark, dull brown: some disintegration	++++	0.9	++++	1.2	Dark, dull red-brown, meat disintegrated: tyrosine crystals
6. B. sporogenes	++	0.3	Dark, dull brown; disintegration	++++	1.2	++++	1.8	Dark, black-brown: marked disintegration; no tyrosine
7. B. welchii and B. aerofoetidus	+++ not blown	0.2	Slight reddish brown	+++	0.7	++++	1.1	Dark reddish brown
8. B. welchii and B. bifermentans	+++ not blown	0.8	Brownish red	++++	1.3	++++	1.5	Brownish, copper red, not disintegrating
9. B. welchii and B. sporogenes	+++ not blown	0.4	Brownish red	++++	1.5	++++	1.8	Dark, brownish red
10. Vibrion septique and B. welchii	++++ meat blown	0.0	Slight pinkish	++++	0.2	++++	0.4	Pink and fluid clouded
11. B. bifermentans and B. sporogenes	+	0.6	Dark reddish brown	++++	1.3	++++	1.7	Dark reddish brown; some disintegration
12. B. bifermentans and B. aerofoetidus	+	0.6	Dark reddish brown	+++	1.2	++++	1.3	Like 4; dark reddish brown
13. B. sporogenes and B. aerofoetidus	+	0.3	Dull dark brown; some disintegration	++++	1.5	++++	1.9	Very dark reddish brown; disintegration
14. B. histolyticus and B. bifermentans	++	0.9	Dull red brown; some disintegration	++++	1.4	++++	1.7	Lighter than 5; no tyrosine crystals
15. B. histolyticus and B. welchii	++++	0.6	Light reddish brown	++++	1.0	++++	1.4	Reddish brown; tyrosine crystals
16. B. histolyticus and B. sporogenes	++	0.7	Like 14	++++	1 5	++++	2.2	Very dark reddish brown; tyrosine crystals
17. B. mesentericus (Subtiloid group)	0	0.0	No change	0	0.0	+	0.0	No change
18. B. mesentericus and B. welchii	++	0.0	Pinkish	++	0.0	+++	0.4	Light copper red
19. B. proteus	+	0.0	No change	+	0.0	+	0.0	Very slight pinkish tinge
20. B. coli	0	0.0	No change	..	0.0	..	0.0	Like 19; slight pinkish tinge
21. B. coli and B. welchii	++	0.0	Slight pinkish	++	0.0	+++	0.1	Like 1; copper red, fluid clouded
22. B. coli and B. sporogenes	Trace	0.0	Dark, dull brown; disintegration like 6	+++	0.8	++++	1.6	Like 6; dark, black-brown; marked disintegration; no tyrosine
23. Control	0	0.0	No change	0	0.0	0	0.0	No change

* Gas symbols: vaseline cap raised to top or out of tube, ++++; raised 4 to 5 cm., +++; 1 to 3 cm., ++; 1 cm. or less, + and trace.

Of the fermentative intestinal spore-bearing anaerobes, B. welchii is by far the most frequently encountered and the most readily recognized. When growing actively in the medium, there is generally such a violent production of gas within 24 hours that the vaseline cap is blown to the top or out of the tube and the meat itself more or less disrupted. The fluid is rather heavily clouded, the turbidity resembling ground glass or diluted milk. Although B. welchii is generally described as nonproteolytic, there occurs nevertheless, within a week, a decrease in the height of the meat column of about 4 mm., possibly due to the digestion of connective tissue. As applied to the analyses of intestinal specimens, if the above changes occur in tubes 1 and 5 seeded with the unheated material within 24 hours' incubation, it is indicative of B. welchii being unusually numerous and actively growing in the intestine. When this finding occurs in the unheated tubes, the reaction typical for B. welchii will generally be noted up to tubes 7 or 9 of the heated series. This is particularly characteristic for pernicious anemia, as has been reported elsewhere,[30] achylia gastrica and is encountered also occasionally in cases of nonspecific colitis. With individuals enjoying normal digestion and on an ordinary diet the highest heated dilution giving the B. welchii reaction is in tube 3, but generally not above 1 (Moench, Kahn and Torrey[30]). With some strains of B. welchii, gas production is not so violent and the vaseline cap may be blown only part way to the cotton plug within 24 hours, but in all instances, the meat assumes the typical reddish copper color. As a further step toward identification, gram-stained films may be prepared from each of the heated suspension seeded tubes showing growth and seedings made from each into milk tubes, using a Pasteur pipet and the usual anaerobic technic and placing a vaseline cap on the milk. The morphology and the milk reaction are adequate for the identification of B. welchii and where the culture is mixed, the identity of the other forms is suggested by the morphology too, one who has had some experience.[31] If complete identification of the types present, other than B. welchii, is desired, it is of course necessary to isolate pure cultures and subject them to suitable differential tests.

Such fermentative members of this group as Vibrion septique, B. oedematiens, B. aerofoetidus, B. fallax, and B. tertius do not cause such pronounced and specific changes in this cooked meat medium as to permit even tentative recognition unless in such unusual numbers as to present a practically pure culture in the higher heated dilution tubes. If such is the case, for Vibrion septique (or B. oedematiens which produces identical changes in this medium) the reaction is somewhat similar to that for B. welchii except that the evolution of gas is less violent, and after a week's incubation, there is no decrease in the height of the meat column, the fluid is not clouded and the meat color is pinkish. The reaction picture for B. aerofoetidus is given in the table and for that produced by B. fallax and B. tertius, reference is made to the recent article by Kahn.[14]

The only bacterial types occurring in the human intestine which produce a definite lowering of the meat column in this medium (0.3 to 0.6 mm.) within 24 hours' incubation are B. bifermentans, B. histolyticus and B. sporogenes. No aerobic bacterium, spore bearing or not, exerts any digestive action on muscle protein—at least such was not noted within the period of this test. Several strains of the B. subtilis-mesentericus group of intestinal origin as well as B. coli, B. proteus and representa-

[31] Weinberg, M., and Sequin, P.: La Gangrene Gaseuse, 1918.

tives of the B. pyocyaneus group were tested under aerobic and anaerobic conditions but without any indication of digestive activity on native protein in the form of muscle tissue. It may be confidently assumed, then, that a marked digestion of the meat indicates the presence of one or more of these anaerobic types in the flora.

As has been stated, the putrefactive propensities of the whole flora are generally best gauged by the decrease in height of the meat column in the tube seeded with dilution 1 of the unheated fecal suspension for although the above types of anaerobes seem to be the only ones which can initiate the decomposition process, there are apparently other bacterial types which may collaborate with them in carrying it further. If after an incubation of 6 to 7 days, there is a lowering of the meat column of from about 1.0 to 1.5 cm., there is indication of a considerable degree of putrefactive tendency on the part of the flora and if more than 1.5 cm., an unusual degree. These figures apply only to examinations of stool specimens, as with material from the small intestine, we may also have the factor of digestive enzymes (Goldman[7]). Occasionally, also, putrefactive floras are encountered in which these anaerobes are practically unrepresented, but the decomposition of the end products of digestion is due mainly to aerobic forms such as B. proteus, B. mesentericus and certain members of the colon group. In such instances there may be little or no digestion of the cooked meat.

The particular type of proteolytic anaerobe, however, which is predominant is best indicated by an inspection of the tubes seeded with the heated (80 C.) fecal suspension dilutions.

B. bifermentans causes the most rapid proteolysis of any type ordinarily found in the intestine. A lowering in the meat column in any of these tubes of over 0.5 cm. within 24 hours with development of some gas is strongly suggestive of its presence, especially, when associated with the rather characteristic yellow, reddish-brown tint of the meat. A further peculiarity is that when unassociated with other proteolytic organisms digestion progresses slowly after 24 hours and does not exceed 1.0 cm. in 7 days, nor is there noticeable disintegration of the meat particles. B. sporogenes and B. histolyticus initiate proteolysis somewhat more slowly than B. bifermentans, but both (especially B. sporogenes) finally achieve a greater degree. Within 24 hours, there is also noticeable a beginning disintegration of meat fibers which becomes very pronounced within the period of the test, together with a marked darkening of the meat, blackening in the case of B. sporogenes and a dark, dull red with B. histolyticus. The latter organism also generally forms white balls of tyrosine crystals within 7 to 12 days. The occasional presence and distribution of B. histolyticus within the human intestine has been described.[32] Of course, in examinations of intestinal material, no one of these spore bearing anaerobes is likely to be found unmixed with other representatives of the group unless it be occasionally B. welchii. The predominant organism is generaly revealed, however, in the highest heated dilution tube showing growth.

[32] Torrey: J. Infect. Dis., 1925, 36, p. 517.

Experiments to determine the action of mixtures of these anaerobes, as shown in the table, has revealed the interesting fact that, although such types as B. welchii and B. aerofoetidus exert very slight proteolytic action when each is growing alone, when grown together digestion is increased 4 to 5 times. Development of either in association with the proteolytic organisms, B. bifermentans, B. histolyticus and B. sporogenes, also definitely increases the rate and degree of digestion. This is particularly the case with the combination of B. welchii and B. bifermentans. It has been my experience that this combination is the one commonly found in the more extreme and objectionable types of intestinal putrefaction with which are often associated symptoms of so-called autointoxication. It may be observed in this connection that B. bifermentans is an active producer of H_2S. These two types seem to thrive much better in the intestine than does B. sporogenes. These tests indicate how B. welchii, a very common habitant of the intestine may enhance putrefaction in the presence of frank proteolytes.

This table also illustrates the temporary inhibitory effect by B. coli on B. sporogenes proteolysis. Streptococcus fecalis has been found to exercise a slightly greater effect and B. acidophilus a decidedly more pronounced action.[33]

The above discussion has applied merely to the spore bearing anaerobes, the isolation of the nonspore bearing strictly anaerobic types is a much more difficult and often a hopeless task. If B. coli and streptococci are not too numerous it is feasible to prepare plate cultures from the unheated dilution 5 meat tube, and incubate them anaerobically or to use the tube dilution method described by Kahn.[30] Such of these types as have been observed by the writer, seemed to be organisms with fermentive rather than putrefactive propensities, but forming only slight amounts of gas. They are apparently of questionable significance as determiners of the character of the flora.

SUMMARY

A group of three cultural procedures are described which collectively form the basis for as comprehensive a bacterial analysis of fecal material as may be desired except for certain specified purposes. This method has the advantage of requiring fewer types of mediums and fewer steps than those recommended heretofore for a similar purpose, and yet the resulting information is more definite and comprehensive.

[33] Torrey and Kahn: J. Infect. Dis., 1923, 33, p. 482.

The cultural elements of the technic include: bromcresol purple lactose agar plates, surface seeded and incubated aerobically; glucose blood agar plates incubated under conditions of reduced oxygen tension; and a series of cooked meat medium tubes with vaseline caps to bring the anaerobic types to development. A standardized method for determining the virulence of members of the colon group is also described.

In the application of this technic to the examination of stool specimens certain points are emphasized:

A reaction more acid than P_H 6.2 or more alkaline than P_H 7.2 is indicative of abnormal bacterial conditions.

Microscopic examination of gram-stained films prepared from stool specimens, except for certain specified purposes, is of little or no value.

An average count for viable B. coli for adults is given and the possible significance of wide departures from this average as well as of unusually virulent colon strains in the intestine is discussed.

The possible relation of various types of streptococcus, such as Streptococcus fecalis, salivarius, hemolyticus, Micrococcus zymogenes and anaerobic types to nonspecific ulcerative colitis and other chronic intestinal disorders is considered in connection with the findings in a series of 132 individuals. The rarity of Streptococcus hemolyticus in the large intestine is emphasized.

A tabulation is given illustrating the differential changes produced in cooked meat medium by the more noteworthy intestinal spore-bearing anaerobes and its use is suggested as a presumptive guide in fecal examinations.

The methods, as described, are adapted particularly to the study of material from the large intestine but with some obvious modifications and additions they may serve as a basis for a survey of the small intestine.

SELECTIVE MEDIUMS IN THE DIAGNOSIS OF RODENT PLAGUE

PLAGUE STUDIES. 1

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The study of a disease in wild rats with gross anatomical lesions resembling plague suggested the use of special mediums which would not only inhibit the bacterium responsible for the infection and the undesirable flora present in the tissues of the decomposed cadavers but selectively enrich Pasteurella pestis. N. E. Wayson [1] has shown that the diagnosis of acute plague in wild rats trapped in certain localities of Oakland was rendered very difficult and confusing by the presence of an organism belonging to the hemorrhagic septicemia group. A bacteriologic confirmation of the routine diagnoses made from inspection of the lesions became a necessity. Cultures prepared on the customary slightly alkaline agar mediums, however, were frequently unsatisfactory on account of the prolific growth of the contaminating intestinal bacteria. Neither the hemorrhagic septicemia nor the plague bacillus could be recognized in the diffusely spreading growth of B. proteus. Animal inoculations were of little value on account of the great virulence of the hemorrhagic septicemia organism. Guinea-pigs infected cutaneously with spleen, liver or lymph node material succumbed to a septicemia in from 18 to 36 hours. It seemed probable that, owing to these conditions (latent hemorrhagic septicemia infection and decomposition), bacteriologic procedures in part and the medium usually employed in the diagnosis of plague might be unreliable and rats infected with P. pestis might escape detection. That this suspicion was fully justified was subsequently proven by the detection of plague in seven wild rats (Wayson 5 rats, and Meyer and Batchelder 2) co-existent with the hemorrhagic septicemia organisms. The laboratory problem was well defined; one or several methods which would inhibit the contaminating organism and eliminate the hemorrhagic septicemia organism had to be devised. Two

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[1] Pub. Health Rep., 1925, 40, p. 1975.

procedures were subjected to an experimental study: preparation of a selective medium, and immunization of guinea-pigs against the hemorrhagic septicemia organism. The latter experiments will be reported in a separate paper. In this connection it must be noted that the methods of immunization, which will protect guinea-pigs against a subcutaneous or "pocket" infection with the pasteurella, are time consuming and therefore impractical in emergencies. As an alternative, the use of mediums, which would inhibit the septicemia organism to such an extent that the plague bacillus would not be overgrown on the agar plate, was therefore investigated. Furthermore, it seemed of interest to carry out experiments with dyes, chemicals, etc., in order to devise a selective liquid medium for P. pestis somewhat analogous in principle to the various procedures now employed in the diagnosis of typhoid-paratyphoid infections.

Aside from the immediate solution of a practical problem it appeared advisable to reconsider the customary bacteriologic methods employed in the diagnosis of rodent plague. The reader of the monographic literature on this subject [Reports on Plague Investigations in India,[2] Dieudonne and Otto,[3] Dunbar and Kister,[4] Report of the International Plague Conference,[5] Williams [6] and others] is impressed by the laconic briefness with which the subject is treated. It is quite evident that some improvements would probably greatly expedite and possibly increase the accuracy of the routine bacteriologic procedures. If a selective medium for P. pestis could be found that would inhibit the intestinal bacteria and enrich the plague bacillus a diagnosis could be given without the use of animals inside of two to three days. A suitable enrichment medium might also furnish positive cultures with rat material that yields negative findings upon inoculation of guinea-pigs owing to advanced decomposition, scarcity or low virulence of P. pestis. In this connection it must be emphasized that the problem of the ultimate eradication of plague in the United States and Europe requires diagnostic procedures which are far more rigid than those usually employed in the control of the endemic foci present on the African and Asiatic continents. In the course of a survey the proportion of the macroscopically poorly marked rats increases as the proportion of infected rats decreases. Since it is the ultimate aim of the public health worker

[2] J. Hyg., 1907, 7, p. 324.
[3] Kolle u. Wassermann: Handb. d. pathogen. Mikroorganismen, 1912, 4, p. 214.
[4] Centralbl. f. Bakteriol., 1904, 36, p. 127.
[5] Report of the Internat. Plague Conference, Manila, 1912.
[6] Am. J. Pub. Health, 1920, 10, p. 861.

to detect the rats which harbor P. pestis without lesions and yet act as virus carriers (Bordas, Dubief and Tanon,[7] Williams,[6] adequate methods should be devised to facilitate the study of such animals. Mass inoculations into guinea-pigs have been used for this purpose, but it is not unlikely that enrichment cultures in selective medium would be more suitable particularly when the chances of picking up a virulent hemorrhagic septicemia organism are greatly increased by the subcutaneous injection of at least ten spleens (Williams and Kammerer [8]).

These considerations lead to the experiments and observations which are the subject of this paper.

The Basic Medium and the General Bacteriologic Procedures.—In a series of preliminary experiments 15 different strains of Pasteurella pestis, several strains of B. coli, B. proteus, Pasteurella muricida were cultured in broth and varying dilutions of an 18 hour culture were either transferred to the liquid held in tubes or streaked on the surface of the solid mediums poured in Petri dishes. Beef heart hormone medium prepared strictly in accordance with the specifications of Huntoon [9] was used, as a foundation. Three peptones, Witte, Berna, and Difco, in 2% amounts were tested and found equally satisfactory. In veal infusion hormone agar or broth Berna peptone favored the development of large colonies of P. pestis in less than 24 hours. The mediums were either carefully decanted or strained through glass wool. A slight precipitate adsorbs the inhibitive dyes, renders the medium less selective but favors the growth of large colonies.

In accordance with the recent studies of d'Aunoy [10] the reaction of the basic medium was adjusted to a P_H of 6.8 to 7.0. The addition of blood or serum or the use of a tryptic-bloodclot-digest hormone medium failed to enlarge the colonies or to render them more characteristic in the chosen incubation time of 18 to 36 hours at 30 C. In fact, these substances reduced the efficacy of the dyes thus making the selective action uncertain and requiring a time consuming preliminary determination of the amount of dye necessary to produce a selective medium. Hormone mediums are in every respect superior to the usual medium prepared by repeated autoclaving and filtration. They gave in 18 to 24 hours an abundant growth of P. pestis which is readily emulsified for serologic studies. When used to preserve stock cultures the virulence is well preserved (observation for 1½ years) on such mediums. The addition of sodium sulphite as recommended by Drennan and Teague [11] is absolutely necessary. It improves the solid as well as the liquid mediums as is well illustrated by the results shown in table 1.

Any medium, even those containing blood or its derivatives, are rendered somewhat inhibitive or toxic for P. pestis and P. muricida when sterilized in the autoclave for 30 minutes at 15 pounds pressure. Only large inoculums grow in form of a fine film composed of relatively small colonies. The hemorrhagic septicemia organism is even more affected than P. pestis. On autoclaved hormone agar seeded with 0.1 cc. of a 1 to 100 dilution of an 18 hour broth culture, small pin head sized colonies appear around the small pieces of beefheart which may remain suspended in the medium. This nursing effect is absent in agar prepara-

[7] Presse méd., 1922, 30, p. 831.
[8] Pub. Health Rep., 1923, 38, p. 1873.
[9] J. Infect. Dis., 1918, 23, p. 169.
[10] J. Infect. Dis., 1923, 33, p. 391.
[11] J. M. Research, 1917, 36, p. 519.

tions containing 0.025% sodium sulphite. A diffuse growth appears on any medium although a veal infusion base rarely furnishes colonies as large as those observed on beef heart hormone agar. Sodium sulphite is probably not the only substance which renders inert the factors contained in the autoclaved agar that inhibit the growth of plague bacilli. The following observations and experiments lend support to this contention. In the course of the many attempts to cultivate plague and pasteurella organisms from cadavers of rats and guinea-pigs on autoclaved agar it was noted that the colonies of these organisms are visible as

TABLE 1

SHOWING THE NUMBER OF COLONIES WHICH GREW ON THE VARIOUS MEDIUMS IN TESTS MADE WITH DILUTIONS OF PURE CULTURES OF P. PESTIS AND P. MURICIDA

0.1 cc. Dilution Plated	Hormone Agar Autoclaved		Hormone Agar Autoclaved with Na_2SO_3		Veal Hormone Autoclaved		Veal Hormone Autoclaved with Na_2SO_4	
	P. pestis	P. muricida	P. pestis	P. muricida	P. pestis	P. muricida	P. pestis	P. muricida
10^{-2}	∞	Few (nursing effect of meat)	∞	∞	∞ Small	∞ Small	∞	∞
10^{-3}	∞	Few (nursing effect)	∞	∞	0	0	∞	∞
10^{-4}	Approximately 200	0	2000±	∞	0	0	∞	∞
10^{-5}	0	0	367	∞	0	0	208	1000±
10^{-6}	0	0	291	950	0	0	122	500±
10^{-7}	0	0	145	700	0	0	0	183

0 indicates no growth, in all tables.

TABLE 2

SHOWING THE NUMBER OF COLONIES WHICH GREW ON THE VARIOUS MEDIUMS IN TESTS MADE WITH DILUTIONS OF PURE CULTURES OF P. PESTIS AND P. MURICIDA MIXED WITH STERILE SPLENIC TISSUES

0.1 Cc. Dilution in Suspension of Rat Spleen Pulp	Hormone Agar Autoclaved		Veal Agar Autoclaved		Plates without Splenic Tissue	
	P. pestis	P. muricida	P. pestis	P. muricida	P. pestis	P. muricida
10^{-4}	∞	∞	∞	∞	0	0
10^{-5}	200±	300±	300±	500±	0	0
10^{-6}	150±	221	280	50	0	0
10^{-7}	124	71	171	0	0	0

large surface discs with undulated (P. pestis) or smooth edges (P. muricida) in those portions of an 18 hour beef heart hormone plate which has been previously streaked with tissue fragments. One of the many experiments presented in table 2 illustrates these findings.

The phenomenon will be the subject of further study and an attempt will be made to correlate it with the more recent knowledge on the growth accessory substances which play a significant rôle in the viability and metabolism of streptococci, pneumococci, etc. In view of the identical action exerted by sodium sulphite upon autoclaved agar with regard to the development of plague bacilli, it is not unlikely that the

tissue particles act on account of certain sulphur compounds similar in nature to those studied by Müller.[12] From a practical point of view the effect of tissues is exceedingly important since the colonies of poorly growing and attenuated strains of P. pestis may be rendered visible by streaking the plates with sterile organ material. On the other hand, it is worth remembering that tissues reduce many dyes and with it the inhibitive effect thus render the medium less selective. Such an action invariably occurs on certain dye-agar plates smeared with tissues, and although the contaminating bacteria are favored, the scattered P. pestis colonies are simultaneously rendered more readily recognizable.

Selective Mediums.—A number of mediums recommended by various workers for the isolation of P. pestis have been tested and modified in order to adapt them to the particular needs of the problem.

MacConkey's Sodium Taurocholate, Neutral Red Medium: The Indian Plague Commission[13] found the sodium taurocholate medium very useful in the differentiation of P. pestis from the organisms of the hemorrhagic septicemia group. The bacterium of swine plague was inhibited on mediums containing sodium taurocholate while P. pestis grew freely. These observations have probably been responsible for the extensive use of bile salt mediums by the English plague laboratories. However, Eastwood and Griffith,[14] in the course of a rat survey, found that the growth of P. pestis was less certain and slower on MacConkey's medium with the addition of lactose and mannitol than on plain agar. The bile salts exerted some inhibitory effect and since they failed to retard the growth of the intestinal organisms, the medium offered no advantages.

A series of experiments have confirmed these various observations. MacConkey's medium, prepared in accordance with the original formula,[15] is a poor substrate for the cultivation of P. pestis in comparison to the hormone agar. The bacterium is usually inhibited; the colonies are scarcely visible before the 30th hour of incubation and they are difficult to identify. In mixtures with B. proteus they are always submerged by the heavy spreading growth.

The basic agar lacks certain accessory nutritive substance for the hemorrhagic septicemia organisms. Plates heavily seeded with P. muricida have remained sterile. To several batches of hormone beefheart, or veal agar, sodium taurocholate was added either alone or in combination with neutral red. The data of such an experiment are shown in table 3. Although the colonies are larger on hormone, taurocholate neutral agar it has been impossible to find a suitable combination of carbohydrates, bile salts and neutral red which would inhibit the rat pasteurella and suppress the intestinal organisms. The medium was therefore discarded early in the course of the work.

Endo medium (Modified): Kister[16] has recommended the use of Endo's agar for the isolation of P. pestis from decomposed rat cadavers. He claims that the medium inhibits B. proteus; however, it is recommended that the proportions of the sodium sulphite and basic fuchsin should be determined by previous tests for

[12] J. Biol. Chem., 1923, 56, p. 158.
[13] J. Hyg., 1908, 8, p. 302.
[14] Ibid., 1914, 14, p. 290.
[15] J. Hyg., 1908, 8, p. 322.
[16] Centralbl. f. Bakteriol., I., O., 1924, 91, p. 280.

each particular lot of medium. Since the basic agar contains sodium sulphite it offers many advantages and has therefore been tested in numerous series. For the sake of brevity one experiment is shown in table 4.

The data in table 4 indicate that 3.0 cc. of a 10% sodium sulphite solution in hormone agar inhibits P. pestis, Pasteurella muricida, B. enteritidis, but allows B. proteus and B. coli to grow into definite small and discrete colonies. When mixed with 0.6% of a saturated alcoholic basic fuchsin solution the plague colonies

TABLE 3

SHOWING GROWTH OF P. PESTIS AND P. MURICIDA ON MACCONKEY'S MEDIUMS COMPARED WITH THAT ON OTHER SUBSTRATES

	P. pestis			P. muricida		
	MacConkey Base	Beefheart Hormone Base and Sulphite	Veal Infusion Base	MacConkey Base	Beefheart Hormone Base and Sulphite	Veal Infusion Base
Plain and lactose..	All, moderate growth when heavily seeded	All, heavy growth	All, small colonies	All, no growth	All, good growth	Definite inhibition
Neutral red, 1% solution, 0.5 cc.						Definite inhibition
Sodium taurocholate, 0.5%						Growth when heavily seeded
Sodium taurocholate and neutral red						Growth when heavily seeded

TABLE 4

GROWTH OF P. PESTIS, P. MURICIDA, B. PROTEUS, B. ENTERITIDIS AND B. COLI ON HORMONE-LACTOSE AGAR WITH SODIUM SULPHITE AND VARYING AMOUNTS OF BASIC FUCHSIN (SATURATED ALCOHOLIC SOLUTION).

	Percentage of 10% Na_2SO_3	Percentage of Saturated Alcoholic Basic Fuchsin	P. pestis Hours			P. muricida Hours			B. proteus Hours			B. coli Hours		
			12	36	66	12	36	66	12	36	66	12	36	63
1	3.0	0.1	0	+	++	0	+	++	Spreading			++	++	++
2	3.0	0.2	0	+	++	0	+	++	Spreading			++	++	++
3	3.0	0.3	0	+	++	+	++	++	Spreading			++	++	++
4	3.0	0.4	±	+	++	+	+	+	Spreading			++	++	++
5	3.0	0.5	+	+	++	+	+	+	++	++	++	++	++	++
6	3.0	0.6	+	+	++	0	+	+	+	++	++	++	++	++
7	3.0	0.7	0	0	+	0	0	+	+	++	++	++	++	++
8	3.0	0	+	++	++	+	++	++	Spreading			++	++	++
9	0	0	0	0	+	0	0	0	0	+	+	0	+	++

++, Large characteristic colonies.

are moderate in size and characteristic; the hemorrhagic septicemia organisms are very small and B. proteus remains confined to colonies provided the plates are seeded lightly. Unfortunately the results have not been uniform when tests in comparative series with field specimens have been carried out. In fact, the Endos agar proved invariably inferior to the gentian violet medium.

Gentian Violet Sodium Sulphite Medium: Drennan and Teague [11] published the first study on the use of a selective medium in the diagnosis of plague. Influenced by the work of Churchman they determined the toxic effect of various dyes on staphylococci and P. pestis in an autoclaved agar medium which had

been improved by the addition of sodium sulphite. In the course of the experiments gentian violet, malachite green, iodine green, basic fuchsin, congo red, saffranin, bismarck brown, night blue, nile blue were found to be nontoxic for the plague bacillus, while Hoffman violet, nile blue, night blue, crystal violet, methyl violet were strongly bacteriostatic for staphylococci. Tests with plague infected cadavers of guinea-pigs, which were allowed to decompose, demonstrated that a nutrient agar containing 0.025% sodium sulphite and 1/700% crystal violet is probably the most effective medium for the selective isolation of P. pestis. A cursory perusal of the literature indicates that the experiments have not been confirmed and the medium has not been tried in the course of routine examinations of rodents.

An extensive series of tests undertaken in this laboratory with pure and mixed cultures and subsequently with field specimens have con-

TABLE 5

Showing Growth of P. Pestis, P. Muricida, B. Proteus, B. Enteritidis and B. Coli on Hormone, Sodium Sulphite, Gentian Violet Agar

	0.25 Cc. 10% Fresh Na_2SO_3 plus Gentian-violet 1:100 Cc.	Growth of Microorganisms														
		P. pestis			Pasteurella			B. proteus			B. enteritidis			B. coli		
		Hours			Hours			Hours			Hours			Hours		
		18	42	66	18	42	66	18	42	66	18	42	63	18	42	66
A 1	0.1	+	++	++	0	+	++	+ colonies			+	+	+	+	+	+
A 2	0.2	+	+	++	0	0	+	+ small colonies			0	+	+	+	+	+
A 3	0.35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
A 4	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A 5	0	+	+	++	+	++	++	Diffuse ++			+	+	+	+	++	++
A 6	0.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
A 7	0	0	+	++	±	++	++	Diffuse ++			+	+	++	+	+	++
B 1	0.1	+	++	++	0	+	+	+	+	+	+	+	+	0	+	+
B 2	0.15	+	++	++	0	0	+	+	+	+	+	+	+	0	+	+
B 3	0.2	+	+	++	0	0	+	±	+	+	+	+	+	0	+	+
B 4	0.25	0	+	++	0	0	0	0	0	±	0	0	+	0	0	+

firmed the conclusions of Drennan and Teague. Malachite green, solid green, iodine green and congo red were far more inhibitive for P. pestis (10 strains tested) than for the contaminating bacteria commonly encountered in the decomposed cadavers of wild rats. However, gentian violet (Coleman and Bell improved) or dahlia (Grübler) added in dilutions not exceeding 1/400% to hormone beef heart agar containing sodium sulphite rendered the mediums quite selective for plague. In order to determine the appropriate proportion of sodium sulphite to gentian violet or dahlia at least 60 batches (10 l. per batch) of hormone beef heart agar have been tested.

The data of two experiments, which illustrate the principles of the tests, are presented in table 5. Before the results are discussed it is deemed important to give some suggestions regarding the preparation of the mediums.

Only fresh solutions of sodium sulphite should be added to the clear melted agar or broth. Accurately prepared 1% alcoholic solutions of gentian violet or dahlia are quite important in order to secure comparative and uniform results with different batches of agar. They are made as follows:

One gm. of gentian-violet or dahlia is triturated in a mortar while adding slowly 100 cc. of 95% alcohol. The solutions, freed from every trace of sediment, are stored in a glass stoppered bottle. Shortly before use 1 cc. of the desired dye solution is diluted in 9 cc. of sterile distilled water (final dilution 1: 1,000) and the desired amounts, 1.5 to 2.5 cc., are added to 100 cc. of the basic mediums containing the sulphite.

For the direct isolation of P. pestis by surface plating a hormone agar which contains 0.025% sulphite and 2.0 to 2.5 cc. of a 1:1000 solution or 1/500 to 1/400% gentian violet is unreservedly recommended. On such plates the spreading growth of B. proteus is inhibited and the development of the pasteurella is retarded for 50 to 60 hours or completely suppressed when incubated at 30 C. Since the hormone agar is slightly more suitable for P. pestis than extract or veal agar, the concentration of the dye may be increased safely over that recommended by Drennan and Teague without preventing the growth of the bacterium.

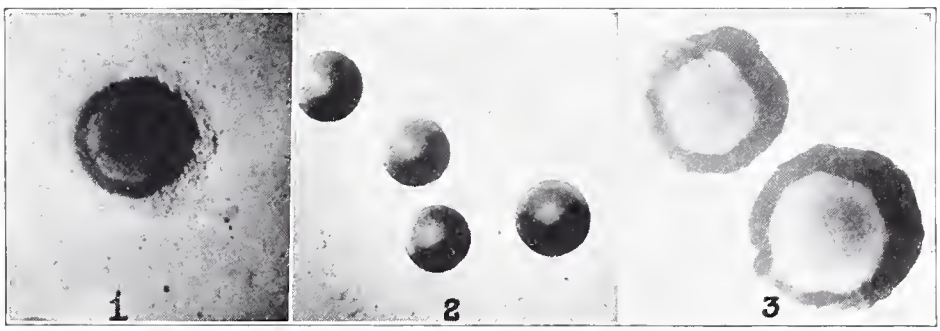

Fig. 1.—Surface colonies on sodium sulphite gentian violet agar, x 18. 1, P. pestis of squirrel origin; 2, Pasteurella muricida; 3, B. pseudotuberculosis rodentium (Pfeiffer).

Neither the sulphite nor the gentian violet alone are capable of restraining B. proteus. However, a concentration of 1/200% of gentian violet without sulphite is bacteriostatic for P. pestis and the intestinal organism employed in the experiments.

The pasteurella organism is more sensitive to gentian violet and dahlia than P. pestis. The direct isolation of this organism from the bodies of animals dead for some days, a concentration of 1/700% of gentian violet (1.5 cc. of a 1:1,000 solution to 100 cc. of agar), is suitable since the contaminating bacteria are restrained to colonies. On incubation for 60 hours a few of the dye tolerant pasteurella organisms develop into colonies. Higher concentration of gentian violet (1/400%) have a strongly bacteriostatic effect unless the plates are smeared heavily with the infected tissues, which exert as already stated a detoxifying effect on the dye. The colonies of the three organisms important in the diagnosis of plague, P. pestis, P. muricida and B. pseudotuberculosis are characteristic (fig. 1) and readily picked with the aid of a

binocular dissecting microscope. The dye is concentrated in the center of the P. pestis while it is usually found in the peripheral zone of the B. pseudotuberculosis colonies.

Gentian violet is equally effective in liquid mediums. Concentrations of as high as 1/400% of the dye will completely inhibit the pasteurella, restrain B. proteus, B. coli, etc., and enrich very few plague bacilli which cannot be demonstrated by direct microscopic examination or by plating. This valuable property has been established by a series of experiments.

Gentian Violet, Series 1: White rats received one or several injections (total 10 cc.) of antiplague serum (Pasteur Institute); they were then cutaneously infected with a virulent strain of P. pestis. As a rule, the animals developed nonfatal, chronic plague infections with definite inguinal and axillary buboes. Five to six weeks after the first infection the nostrils of the rats were touched with a loopful of a culture of the pasteurella organism. Usually the second infection proved fatal in 36 to 72 hours. The blood stream was found teeming with bipolar, coccoid bacilli. Plates streaked with the tissues (buboes, spleen, liver) gave an abundant growth of the hemorrhagic septicemia organism; P. pestis was culturally not demonstrable or developed in few noncharacteristic colonies. However, enrichment in gentian violet broth (2 to 4 days at 30 C. or 10 days at room temperature) invariably developed positive cultures of P. pestis when a loopful was plated. The advantages of the enrichment were even more striking when the cadavers of the rats were kept at room temperature for several days. Direct plating on gentian violet agar often gave negative cultures for P. pestis, and the pasteurella organism which is rapidly destroyed in decomposed tissues, while the organs incubated in dye broths and subcultured on solid mediums furnished plates from which plague colonies could be regularly fished. The efficacy of the gentian violet agar or broth medium is further shown by the following interesting observation.

A cadaver of a guinea-pig experimentally infected with plague was kept in a closed glass jar at a temperature of from 10 to 18 C. During the first month at weekly and later at monthly intervals, the decomposed tissues were cultured for P. pestis. Irrespective of the highly putrid character of the specimens, the plague bacillus was isolated by direct plating for eight months. At that time the soft tissues had completely disintegrated into a brownish, syrupy mass and large specimens had to be smeared. On the ninth and tenth months the dye plates showed no growth. However, a sample, the size of a small cherry, enriched in hormone, gentian violet, sulphite broth at 28 C. showed on the eighth day of incubation practically a pure culture of a virulent P. pestis. Subsequent attempts to isolate the plague bacillus during the 11th, 12th and 13th month yielded negative findings.

It has been repeatedly shown that P. pestis remains virulent in decomposed cadavers. Zlatogoroff,[17] who probably conducted the most detailed experiments on the persistence of plague bacilli in cadavers of plague infected guinea-pigs, found that the temperature at which the bodies were held was of greatest importance.

Cadavers held at a temperature of 12 to 15 C. showed P. pestis for 28 days, and at 4 C. for 102 days. Maassen[18] and Otto report similar results; in fact Otto[19] concludes that the plague bacilli cannot remain virulent in decomposed

[17] Centralbl. f. Bakteriol., I. O., 1904, 36, p. 559.
[18] Arb. a. d. k. Gsndhtsamte, 1902, 19, p. 508.
[19] Festschr. z. 60. Geburtst. v. Robert Koch, Jena, 1903, p. 331.

cadavers for more than two months. Strong in his summary of the bacteriology and pathology of pneumonic plague before the International Plague Conference in Mukden states (5, p. 446) that "in putrid animals, the plague bacillus usually does not live longer than one week, but in one instance this organism was found to be present one month after death." The presence of virulent plague bacilli was established by subcutaneous, cutaneous or pernasal injections or by feeding of the infected putrid tissues on guinea pigs or rats. It is not unlikely that considerably longer survival times would have been recorded had some method of enrichment been employed.

TABLE 6

SHOWING THE NUMBER OF P. PESTIS WHICH GREW FROM INFECTED SPLEENS OF RATS ON VARIOUS MEDIUMS

	Fresh Spleen					Decomposed Spleen			
	Number of P. pestis per Plate per Cc.			Enrichment in Dye Broth 48 Hours Incubation	Results on Guinea-Pigs	Number of P. pestis per Plate per Cc.		Enrichment in Dye Broth 48 Hours Incubation	Results on Guinea-Pigs
Dilution of Splenic Pulp	Plain Agar	Plain Agar with Na_2SO_3	1/400 % Gentian Violet Agar			Plain Agar with Na_2SO_3	1/400 % Gentian Violet Agar		
10	∞ (24 hrs.)	∞ (24 hrs.)	∞	—	—	Diffuse spreader	Spreader	—	—
10^{-1}	∞ (24 hrs.)	∞ (24 hrs.)	∞	—	—	Diffuse spreader	∞ Plague	—	—
10^{-2}	370 (24 hrs.)	∞	∞	—	—	Diffuse spreader	Contaminator confined ∞ plague	—	—
10^{-3}	0	∞	∞	—	—	Contaminator restricted 60 (48 hrs.)	4700 Plague	—	—
10^{-4}	0	3690 (48 hrs.)	5300 small	—	—	Contaminator restricted plague present	5900 Plague	—	—
10^{-5}	0	5290 (48 hrs.)	1000 (48 hrs.)	∞	—	±3400 (48 hrs.)	1880 Plague	—	—
10^{-6}	0	320 (48 hrs.)	260 (48 hrs.)	∞	—	2810 (48 hrs.)	2810 Plague	∞ Plague	—
10^{-7}	0	0	110 (48 hrs.)	∞	Alive	1090	3400 Plague	∞ Plague	Alive
10^{-8}	0	0	0	∞ (agglutination)	Alive	0	210	∞ Plague	Alive
10^{-9}	0	0	0	0	Alive	20	60	∞ Plague	Alive

To prove conclusively the excellent properties of gentian violet mediums in the diagnosis of plague, a series of experiments have been conducted. Since the results are significant, the tests are given in detail.

Series 2: Two white rats were infected with a laboratory strain of P. pestis (squirrel strain); one was sacrificed on the fourth day after the injection. Typical plague lesions were present. The spleen was removed aseptically. The second rat died on the fifth day; the cadaver was kept at 37 C. for two hours. The spleen of the first and of the second rat were triturated separately in mortars and suspended in sterile salt solution in the proportion of 1 part of spleen to 10 parts of saline. Serial dilutions were made, 0.1 cc. was streaked on the surface of different agar mediums; 1.0 cc. was enriched in gentian violet broth or injected subcutaneously into guinea-pigs. The results are shown in table 6.

The experiments fully illustrate the value of the gentian violet plates as an excellent medium for the isolation of P. pestis. The enrichment cultures showed the plague bacillus in one of the highest dilutions which gave sterile gentian violet plates. Since the guinea-pigs given 1 cc. of several of the highest dilutions remained uninfected it appeared advisable to repeat the experiment with a virulent plague strain. The observations which will be detailed below have been made with a highly pathogenic, recently isolated strain of squirrel origin.

Series 3: The spleen of a white rat experimentally infected with plague material was triturated and suspended in salt solution. Serial dilutions were streaked on gentian violet, sulphite agar or enriched in gentian violet broth;

TABLE 7

Showing the Number of P. Pestis Which Grew From Infected Spleens of Rats and the Effect of Ground Splenic Tissues on the Virulence of the Plague Bacillus

	Fresh Spleen			Decomposed Spleen		
Dilution of Splenic Pulp	Number of P. pestis on Gentian Violet, Sulphite Plates per Cc.	Enrichment in Gentian Violet Broth	Results of Guinea-Pig Inoculations: Day of Death	Number of P. pestis on Gentian Violet, Sulphite Plates per Cc.	Enrichment in Gentian Violet Broth	Results of Guinea-Pig Inoculations
10	∞	+	Sacrificed 9th, plague	— ∞	+	Sacrificed 9th day
10^{-1}	∞	+	Sacrificed 9th, plague	— ∞	+	Sacrificed 9th day
10^{-2}	∞	+	Sacrificed 9th, plague	— ∞	+	Sacrificed 9th day
10^{-3}	∞	+	Sacrificed 9th, plague	— ∞	+	Died on 4th day
10^{-4}	∞	+	Sacrificed 9th, plague	— ∞	+	Sacrificed 9th day
10^{-5}	6270	+	Died: 4th	Approximately 9100	+	Died of plague 5th day
10^{-6}	590	+	3rd	1810	+	3rd day
10^{-7}	80	+	5th	210	+	3rd day
10^{-8}	20	+	5th	60	+	5th day
10^{-9}	0	+	7th	0	0	7th day
10^{-10}	0	—	Not infected	0	0	Sacrificed, not infected

one cc. of each dilution was inoculated subcutaneously into a series of guinea-pigs of the same weight. The same procedures were repeated with a spleen which was removed from an infected rat sacrificed on the third day of the disease and incubated for seven hours at 37 C. The results of this test are summarized in table 7.

As a whole the series confirmed the preceding experiment. The enrichment cultures of the triturated fresh spleen indicated the presence of P. pestis in a dilution which gave sterile gentian violet plates and thus supported the positive guinea-pig findings. In the cultural series with decomposed splenic tissues the animal inoculations proved slightly more sensitive than the enrichments. However, the experiments revealed a new and exceedingly interesting fact.

The guinea-pigs inoculated with the dilutions 1 : 10, 1 : 100, 1 : 1,000, 1 : 10,000 and 1 : 100,000 appeared ill, but recovered and were sacrificed on the ninth day after the injection. They showed typical lesions of plague but extensive cultures of the organs of several animals remained sterile. On the other hand the anatomical, microscopical and cultural tests of the guinea-pigs (injected with the dilutions 1 to 1 million to 1 to 10 billion) which died on the 3rd to 6th day were uniformly positive for acute plague. In order to verify these paradoxic results several additional series, the details of which will be published elsewhere, were prepared. With absolute constancy the guinea-pigs injected with the lowest dilutions of the ground infected splenic tissues recovered from the plague infection, while those treated with the highest dilutions, some receiving less than 10 bacilli died acutely of plague in less than five days. The addition of splenic pulp to diluted broth cultures gave similar results. More recently some experiments have been conducted with liver and lymphnode material. No curative effect of these tissues has been noted. The animals injected with the lowest died just as rapidly as those treated with the highest dilutions. It is therefore evident that the spleen of the white rat possesses a property or properties which reduce the virulence of the plague bacillus. Without further studies it would be unwise to speculate concerning the nature of the phenomenon. Attention is merely called to the fact that the experiments unexpectedly prove the advantages of the direct or the enrichment cultures of plague spleens over the animal tests. Those, who have access to spontaneously infected rat cadavers, should conduct such resistance and viability tests on a broad scale by comparing the guinea-pig inoculation test with the gentian violet broth enrichment method. The development of an economical and rational bacteriological diagnosis of plague in fresh and decomposed rodents depends on the outcome of such experiments.

In a preceding paragraph it has been stated that dahlia can be used as a substitute for gentian violet, but since the latter gave more uniform results and is probably available in every laboratory, it has received preference in the practical tests which are reported below.

Gassner's Metachrome Yellow-Water Blue Medium.—In the course of some experiments, which were made in order to purify several strains of P. pestis, some of the dye mediums generally used in the diagnosis of typhoid-paratyphoid infections were employed. Quite accidentally it was found that the medium of Gassner,[20] 100 cc. of hormone agar (P_H 7.0), 5 cc. of 20% lactose solution, 6.0 cc. of a 2% aqueous solution of metachrome yellow (Alizarin Yellow GG—) and 8.0 cc. of a 1% aqueous solution of water blue inhibited the pasteurella organisms completely. For the isolation of the plague bacillus from decomposed cadavers the medium is unsuitable; B. proteus, B. coli, etc., develop freely and frequently

[20] Centralbl. f. Bakteriol., I.O., 1918, 80, p. 219.

submerge the more slowly growing P. pestis. The medium in liquid form is, however, of practical value in the identification of the latter organism. The plague bacillus forms a fine pellicle or yellowsh ring and flakes along the test tube wall; it does not change the greenish color, while the paratyphoid bacilli produce a diffuse turbidity with a slight clearing of the medium. The lactose fermenters turn it blue and generate gas. The medium is superior to that recommended by Uriarte and Moralles-Villazon.[21]

PRACTICAL APPLICATION OF THE SELECTIVE MEDIUMS

The remarkable selective properties of the gentian violet, sulphite agar and broth has been proven in the course of a series of tests with wild rats, and with squirrels submitted for a plague diagnosis and in connection with some immunity experiments with P. pestis, and with B. pseudotuberculosis. A brief account of these tests is given in the paragraphs which follow.

With Wild Rat.—As soon as the quantitative experiments had established the value of the gentian violet medium, it was used in the cultural examination of 50 rats. The pathologic findings of the animals, which were received from the U.S.P.H.S. Plague Eradication Laboratory in San Francisco, will be reported in a subsequent paper. In order to diagnose the microorganism responsible for the lesions in the liver, spleen, lung, etc., guinea-pigs and rats were inoculated either by the cutaneous, subcutaneous or nasal route. Simultaneously a set of plain and of gentian violet, sulphite agar plates were inoculated with portions of the organs. Pieces of the tissues or exudates (spleen, liver, lung, buboes), heart blood or a few drops of the pericardial and the pleural effusion were used. The method finally elaborated follows:

By dipping the organs into paraffin oil held at a temperature of 180 C. for two to five seconds the surfaces are sterilized. With a sterile scalpel they are divided and a clean section is streaked over the agar. Badly contaminated specimens are ground with sand and dilutions of the pulped organs are spread on the medium. The plain agar plates are held for 18 hours, the gentian violet plates for 24 hours at 30 C; with the aid of a binocular dissecting microscope, they are searched for characteristic colonies. In case the tissues of fresh cadavers are cultured, typical colonies are readily picked from the plain agar plates inoculated with material from the buboes. Spleen and liver cultures on the same agar are quite often covered by spreaders so that the detection of the diagnostically important colonies is mostly impossible. On the other hand, the dye sulphite plates with 1.5 cc. of a gentian violet dilution 1:1,000 in 100 cc. of agar restrain the contaminators. The large colonies, which develop on plates incubated for 48 to 72 hours, emulsify readily in salt solution or serum dilutions and are often rapidly identified by slide agglutination tests. P. pestis and P. muricida grown on dye agar rarely exhibit spontaneous clumping. The procedures just outlined are not applicable to the plain agar plates. The colonies, if they are not overgrown by 18 hours of incubation at 18 C., are small and usually mucoid. Prolonged incubation at 15 or 30 C. furnishes plates, which for the purpose of cultural discrimination, are unsatisfactory. Colonies may be picked but the transplants are mixed cultures and mostly unsuitable for agglutination or biochemic tests.

The cultural tests obtained with the organs of 50 rats on two mediums and compared with the results of the animal inoculations

[21] Compt. rend. Soc. de biol., 1924, 91, p. 1041.

(table 8) show that the tissues of 28 wild rats harbored the organisms of hemorrhagic septicemia, which were recognized by direct plating on gentian violet agar. In fact, in two animals the cutaneous vaccination of splenic tissues failed to infect the guinea-pigs but the dye plates demonstrated the presence of the pathogenic bacterium. Too much importance should not be placed on the negative outcome of the animal inoculations. It is not unlikely that subcutaneous injections of the tissues might have indicated the nature of the infection in the wild rat. However, such a procedure would have been very risky on account of the advanced state of decomposition in which the cadavers were received. The fact that the plain agar plates established the diagnosis in only one-half of the

TABLE 8

DIAGNOSIS OF 50 WILD RATS

Infection and Number of Rats Examined	Diagnosis Established by			
	Animal Inoculations	Culture on Plain Agar	Gentian Violet, Sulphite Agar (1/700 and 1/400% Dye)	Remarks
Pasteurellosis 28.....	26	13	28	
Plague 3.....	1 (1 pasteurellosis)	0 (overgrown)	2	One by direct plating
Pseudotuberculosis 1	1 (1 pasteurellosis)	0	1	

infections proven by other methods clearly attests to the superiority of the gentian violet medium. The experiences with P. pestis and B. pseudotuberculosis rodentium (Pfeiffer) are too small to justify any final claims. However, it must be emphasized that two mixed infections of P. pestis and B. pseudotuberculosis with the hemorrhagic septicemia organism were recognized on the dye plates although the animal inoculations suggested a simple pasteurellosis. The significance of these results as far as the diagnosis of P. pestis is concerned has already received repeated recognition in this paper, but the demonstration of B. pseudotuberculosis by direct culture from the organs of wild rats deserves consideration. Neither the monograph of Poppe [22] nor the many brief accounts, which deal with the organism of pseudotuberculosis, mention the occurrence of this organism in wild rats. In fact, the impression is universal that it may confuse the diagnosis of plague when guinea-pigs inoculated with the suspected material are suffering from latent pseudotuberculosis infection. The Indian Plague Commission [23] thus reports that "pseudotuberculosis has never been encountered in the stock of guinea-pigs used in Bombay. An epizootic or even the occurrence of sporadic cases would seriously complicate the use of the

[22] Kolle u. v. Wassermann: Handb. d. pathogen. Mikroorganismen, 1913, 5, p. 775.
[23] J. Hyg., 1907, 7, p. 356.

cutaneous or subcutaneous method in the diagnosis of rats suspected of being plague infected." The direct isolation of this bacterium on gentian violet plates streaked with splenic material of a norvegicus rat confirms the findings of Petrie and Macalister [24] and leaves no doubt that these rodents may suffer from spontaneous infections due to the B. pseudotuberculosis rodentium (Pfeiffer). This interesting fact deserves further study and proper recognition.

With Ground Squirrel Specimens.—Dr. N. E. Wayson isolated P. pestis from putrid plague lesions of ground squirrels by direct plating on gentian violet agar. The plain agar plates were completely overgrown. A final diagnosis was rendered three days before the "vaccinated" guinea-pigs died. Their tissues were badly invaded by putrefactive organism and the detection of the plague bacillus on plain agar plates was impossible. The writers isolated P. pestis on gentian violet plates from the spleen, lymph nodes and necrotic putrid skin of these guinea-pigs and confirmed the diagnosis by macroscopic agglutination tests in 72 hours.

Isolation of P. pestis by Enrichment.—The value of the gentian violet, sulphite broth for the enrichment of P. pestis is shown by two striking observations.

Enrichment of Attentuated Strains: Two guinea-pigs were infected with a strain of P. pestis recently received from the Haffkine Laboratories at Bombay. The animal, which received 0.1 cc. of an 18 hours old broth subcutaneously, developed a large, left inguinal bubo; the guinea-pig vaccinated with the same culture showed no symptoms. Forty days after the inoculation had been made the rodents were chloroformed. The animal injected by the subcutaneous route showed a hard indurated inguinal lymph node and a scar in the liver; the anatomical picture resembled that of a chronic resolving plague infection. Gentian violet agar plates heavily streaked with material from the bubo, spleen, liver, lung and heart blood remained sterile. Pieces of the same organs enriched in dye broth furnished in five days a pure culture of P. pestis in the tube containing the lungs and the spleen. The strain was readily agglutinated but infected guinea-pigs only on subcutaneous inoculation in massive doses. The guinea-pig, which had been infected by cutaneous vaccination, showed no lesions at autopsy. The plate cultures remained sterile, but positive lung cultures were obtained by enrichment. The same result was noted in a similar experiment.

Enrichment of a Few P. Pestis: Five rats received a total of 5 cc. of a highly potent antiplague serum. Three animals were infected by the subcutaneous route and in two the skin was punctured with a fine needle charged with a young broth culture of P. pestis. Both animals were ill for a few days, developed small buboes but recovered while the three subcutaneously infected guinea-pigs died on the fifth day with typical lesions of plague. On the 40 days after the infection the surviving two guinea-pigs were sacrificed and examined. The negative post mortem findings lead to intensive cultural tests. Dye plate cultures remained sterile, but by enrichment of the lung and spleen a fine granular deposit

[24] Rep. to Local Gov. Board on Pub. Health. New Series No. 52, 1911, p. 56.

was formed on the walls of the dye tubes and a pure culture of a virulent P. pestis was isolated by transplantation on solid mediums.

Isolation of B. Pseudotuberculosis from the Lungs of White Rats Experimentally Infected with this Organism: In the differential diagnosis of B. pseudotuberculosis from related organism, white rats are normally inoculated and since the animals exhibit neither clinical symptoms nor pathological lesions it is generally believed that this species of rodent is non-susceptible to this organism. Enrichment in dye broth, however, proved this conception to be erroneous. Ten rats were injected subcutaneously and intraperitoneally with 0.1 cc. of a young culture of an organism which corresponded biochemically, serologically and by guinea-pig tests with the published descriptions of B. pseudotuberculosis rodentium. An equal number of wild rats injected at the same time died from the infection and presented at necropsy extensive changes in the liver and spleen. The white rats behaved like normal animals, they were sacrificed on the 25th day. No lesions were noted, although the enrichments of the lung lobes of each rat gave pure cultures of the inoculated organisms.

The observations which indicate the great usefulness of the gentian violet broth in the diagnosis of plague and allied rodent infections need further confirmation. They have suggested a number of important problems. For example, the latency of P. pestis in the tissues of rats deserves renewed cultural investigation. One cannot escape the impression that the constant demonstration of attentuated or virulent plague or pseudotubercle bacilli in the lungs of guinea-pigs and rats may perhaps be of some significance in the pathogenesis and distribution of the disease. Those engaged in plague control work will also ask the questions: Is the spleen of decomposed rats the best organ for cultures? Can the diagnosis be facilitated by enrichment cultures of lung tissues? These and many other questions have not been in the scope of the present investigation, but they will receive consideration as soon as suitable specimens are available.

CONCLUSIONS

An autoclaved hormone beef heart, or veal Berna peptone agar prepared according to the formula of Huntoon is rendered most suitable for the cultivation of P. pestis by the addition of .025% (.25 cc. of a freshly prepared 10% solution) sodium sulphite and 1/400% (2.5 cc. of a 1:1,000 solution per 100 cc.) of gentian violet (improved Coleman Bell). Such a medium inhibits the most troublesome contaminators usually encountered in diagnostic work on rats and squirrels. The large colonies, which develop in 36 to 48 hours, are characteristic and may be identified by slide agglutination tests. The advantages of a gentian violet enrichment broth for the detection of plague without lesions are discussed.

A DISEASE IN WILD RATS CAUSED BY PASTEURELLA MURICIDA, N. SP.

PLAGUE STUDIES. 2

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The plague eradicative measures recently instituted in various localities on the Pacific Coast supplied an abundance of specimens which offered an excellent opportunity to study the various spontaneous rat diseases transmissible to man. In collaboration with Dr. N. E. Wayson, in charge of the Plague Control Laboratory, U. S. P. H. S., at San Francisco, an estimation of the percentage of paratyphoid-enteritidis infections among the wild rats caught in the San Francisco Bay region was at first contemplated. However, another problem very much more important from a practical point of view forced this study temporarily in the background. Wayson [1] observed during the routine examination of wild rats a disease the gross pathology of which resembles acute or resolving plague. A preliminary study of a few cases of this disease suggested an infection with an organism of the hemorrhagic septicemia group.

In order to establish simple criteria which would enable the rapid differentiation from P. pestis or B. enteritidis infections it became necessary to make a careful comparative pathologic and bacteriologic study of a series of rats with lesions simulating plague. The various findings which have been made will be considered separately. In this paper the infections due to the bacteria of the hemorrhagic septicemia group are described.

Laboratory Procedures.—The rats which supplied the data incorporated in this publication were selected from a survey of 32,000 rats. As a rule animals which exhibited some lesions, or lesions characteristic of acute, subacute or resolving plague, were segregated in the Plague Control laboratory from the majority of the rats with negative post mortem findings, by two groups of experienced workers. These "suspicious" rats were scrutinized by a third group and the cadavers which were in a fair state of preservation were taken to the laboratory. Careful and detailed necropsy notes were taken; smears were prepared from the exudates, spleen, lymph nodes, liver, lung, heart blood or special lesions. They were stained

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[1] Pub. Health Rep., 1925, 40, p. 1975.

by Wayson's [1a] and by Gram's method. As a rule pieces of the spleen and liver, lung or bubo were introduced by the pocket method, or by vaccination (cutaneous method) into guinea-pigs and white rats. Surface cultures were made on gentian violet, sulphite, hormone agar plates.[2] Enrichments were secured in gentian violet or brilliant green, gentian violet broth, and subcultures were made on gentian violet or brilliant green, eosin agar. The tubes and plates were kept at 30 C.; they were examined daily with the aid of a binocular dissecting microscope. A number of colonies (illustrations [2]) were transferred to veal infusion agar slants or identified by slide agglutination tests. The animals which succumbed to the inoculations were carefully examined and the tissue specimens were treated in the same manner as the original material. The guinea-pigs or rats which survived were chloroformed on the 30th day and cultured.

The stock cultures were kept at room temperature in semisolid veal infusion hormone agar, or in heated rabbit or rat serum stratified with vaseline since it was found that at room temperature less frequent transplants were needed to preserve the strains. When kept in the ice chest they gradually lost their pathogenicity or died out.

In table 1 are summarized the final diagnoses which are based on the pathologic and bacteriologic studies of 88 wild rats. Although the actual number of the animals studied is small, it can nevertheless be considered as a representative of the main rat diseases which were encountered in certain localities of Oakland during the months of January to July, 1925. In fact, approximately one-fifth of the total number (320) of the wild rats which exhibited lesions characteristic of plague have been tested by animal inoculations, cultures or both. Unfortunately various procedures such as special culture mediums, the immunization of guinea-pigs, etc., had to be developed and some results were probably lost by laborious but unreliable methods. For this reason the course of the hemorrhagic septicemia epidemic was not followed very carefully. The available data merely indicate that the disease was less frequently encountered as the survey progressed and the eradicative measures reduced the rat population. Furthermore, the character of the lesions changed and it became evident that the disease or diseases involved became more chronic. With the simple and the rapid methods now available the same investigation could be extended to every suspicious rat and therefore a larger portion of a survey could be covered. That interesting and important observations will be made is clearly indicated by the findings here reported.

[1a] Wayson's stain: Fuchsin, 0.2 Gm.; methylene blue, 0.75 Gm.; acid carbolic 99.5%, 10.0 cc.; distilled water, 200.0 cc.; absolute alcohol, 20.0 cc. Dissolve fuchsin and methylene blue each in the absolute alcohol. Dissolve separately the phenol in the distilled water. When completely dissolved add the dye solution to the phenol water; filter before storing for use. The solution will not overstain and acts in a few seconds.

[2] Meyer and Batchelder, J. Infect. Dis., 1926, 39, p. 370.

TABLE 1

PATHOLOGY OF 88 WILD RATS. POSITIVE FINDINGS

Wild Rats					Liver		Spleen			Pleural Cavity			
Bacteriologic Findings	Number	Subcutaneous Injection	Brick Red Colored Muscles	Enlarged and Injected Lymph Nodes	Enlarged	Necrosis	Enlarged	Necrosis	Effusions	Congestion of Lungs	Pneumonia	Bronchiectasis	Pericarditis
Animal inoculations and cultures demonstrated pasteurella organism	36	31	14	30	27	16	34	1	3	6	17	1	2
Animal inoculations or cultures demonstrated pasteurella and B. pestis	1	1 (extensive)	—	1 inguinal bubo	1	—	1	1	1 (extensive)	—	—	—	—
Cultures, B. pestis from lymph nodes	1	—	—	1 bronchial	—	—	1	—	—	—	1	—	—
Cultures, B. enteritidis.....	8	2	—	1	5	4	8	5	1	2	—	2	—
Animal inoculations and cultures demonstrated pasteurella and B. enteritidis	1	1	—	—	1	1	1	1	1	—	—	—	1
Animal inoculations demonstrated pasteurella and B. pseudotuberculosis	1	1	—	1 (hard)	1	1(scar)	1	—	—	1	—	—	—
Animals died following inoculations of spleen or liver tissues; cultural tests negative (putrid cadavers)	2	2	1	1	2	—	2	—	1	—	1	—	1
Animal and cultural tests negative	22	10 (11 very slight)	1	13 (2 slight)	19	12	21	4	—	2	4	2	—
Normal rats	17	—	—	—	—	—	—	—	—	—	—	—	—

A glance at table 1 reveals that four diseases existed among the rats in Oakland: hemorrhagic septicemia or pasteurellosis, plague, rat typhoid and pseudotuberculosis. The last three infections were not infrequently complicated by a superimposed invasion with the hemorrhagic septicemia organism. Since this is the first extensive hemorrhagic septicemia epidemic which has been encountered in a survey of wild rats, it appeared of interest to establish first the lesions which are characteristic for the uncomplicated pasteurellosis and secondly determine the differential diagnostic features which distinguish this disease from the other three rat maladies. The lesions of 36 rats which were proven by microscopic examination of tissue smears, by inoculations of guinea-pigs or rats, or by cultures on gentian violet (1/700%), sulphite

TABLE 2

BODY AND SPLEEN WEIGHTS OF NORMAL AND DISEASED WILD RATS

	Hemorrhagic Septicemia		Normal	
	Weight, Gm.		Weight, Gm.	
	Body	Spleen	Body	Spleen
	210	3.5	310	2.2
	300	4.0	215	1.5
	300	3.9	261	1.5
	200	3.0	208	1.3
	285	3.8	305	3.0
Average	259	3.64 (1/70 body weight)	259	1.9 (1/140 body weight)

agar to be caused by an uncomplicated infection with a hemorrhagic septicemia organism will be discussed according to the records partially summarized in table 1.

Necropsy Findings on Wild Rats.—Thirty-four (94.1%) of the rats examined were three fourths or fully grown and belonged to the Norway variety. Twenty-seven of the young and old rats came from one particular garbage dump near the Oakland Terminus, while the remainder, mostly young animals, originated from rat centers near fertilizer plants, slaughter houses, tenement houses and dumps in Oakland or Alameda. As a rule the cadavers were in a good state of nutrition. Rats which exhibited pulmonary lesions were definitely emaciated.

Subcutaneous Congestion and Edema: The subcutaneous tissues were involved in 31 rats (86.1%); in five animals a gelatinous, yellowish edema was found in the submaxillary spaces. The blood vessels on the abdominal, thoracic, axillary or cervical regions were deeply injected and sometimes surrounded by an area of purplish imbibition or definite hemorrhages. The hemorrhages were more often recorded in the young than in the old rats.

Lymph Nodes: The same animals showing subcutaneous congestion, presented in any region of the body enlarged, soft, or tough lymph nodes, the size of small seeds, surrounded by a radiating net work of dilated blood vessels and in a few instances a moderate oedematous, whitish infiltration. As a rule the nodes were not adherent but the capsule was occasionally thickened and enclosed grayish to deep reddish, medullary centers; necroses were not observed. On pressure the cytoplastic tissue was in some cases readily squeezed out. Primary buboes with yellowish, creamy centers as seen in plague were not recorded in the uncomplicated cases of the infection.

Abdominal Organs: The liver was definitely enlarged, friable and congested in 34 animals (94.1%); the color varied from a deep brown to a mottled nutmeg appearance. In one-half of the specimens small necrotic foci were scattered over the surface and throughout the liver substance. The gay and whitish granules were usually very discrete, not larger than the size of a pin's point and rarely gave the organ the stippled appearance characteristic for plague. It may be mere coincidence but the "granular" condition of the liver was faintly marked in the rats which had pneumonic lesions.

The spleen was always enlarged, "plump," dark brown to purplish in color with rounded edges and pronounced moulded appearance. In the majority of cases the organ was twice as heavy as that of normal rats.[3] This statement is well illustrated by the data presented in table 2.

In the firm but friable pulp the malpighian bodies were usually prominent; necrotic foci were noted in one animal only. In three rats a perisplenitis connected the organ with the viscera and the abdominal wall. The kidneys were usually congested and exhibited in some animals parenchymatous changes. The serous covering of the intestines was moist but no peritoneal exudates were noted. The stomach and the intestines showed no characteristic changes.

Thoracic Organs: The presence of pleural effusions so characteristic for plague was recorded in 3 necropsies (8.3%). In two cases it was abundant, clear, and slightly straw colored, in the others it was moderate, slightly blood tinged and contained flakes of fibrin. In these cadavers the lungs were completely collapsed and grayish in color.

The lungs of over one-half of the rats presented the lesions of a pneumonia which was lobar or lobular in character. The right and left ventral or the anterior portions of the caudal lobes were involved. The consolidation was usually but not regularly symmetrical. The involved tissues were voluminous and varied from a flesh red or dark red to a variegated grayish red. The grayer the tissue the firmer it was. The pleura was covered in two instances by a thick layer of fibrin. On section a gray, yellowish exudate was always present in the larger bronchi; portions of the consolidated lungs sank in water. The mucosa of the trachea was frequently injected. All stages of red and gray hepatization and resolution were present in 10 rat cadavers and as a rule the cephalic lobes showed the more advanced stages of consolidation. Yellowish necroses surrounded by deep brownish demarcations as seen in plague were not observed. The pneumonic lesions which may be considered pathognomonic for hemorrhagic septicemia can be readily differentiated from the patchy congestion or hemorrhages which were found in about 15 % of the necropsies. In one animal the right cephalic lobe was transformed into a yellowish tumor-like mass which contained several large bronchiectases surrounded by areas of partially resolved pneumonia. The pus found in the dilated bronchi was teeming with coccoid bacilli.

The pericardial sac contained in two rats a yellowish exudate with a few strands of fibrin. The serosa showed in several instances small petechiae. The myocardium was soft and was "tigered" in six cases.

From the gross appearances, from sections and from the inoculation experiments in which the anatomic picture was reproduced, the inference may be drawn that rats in various stages of the disease reached the dissecting table. In recapitulation the following postmortem findings may be considered as characteristic of uncomplicated hemorrhagic septicemia infections.

Characteristics of Acute Form: Marked localized or diffuse injection of the subcutaneous tissue; oedema in the submaxillary spaces; brick red color of the muscles; slightly enlarged moist lymph nodes; hyperemia of the liver, enlargement and hyperemia of the spleen, congestion of the lungs, perhaps pleural effusion.

Subacute or Chronic Form: Moderate to extensive injection of the capillaries around the enlarged, firm, nonadherent lymph nodes; large, yellow brownish, fatty liver with numerous necroses, splenic tumor, occasionally with perisplenitis and adhesions; extensive red and gray lobar consolidation of the cephalic and anterior portions of the principal lobes; tracheo bronchitis; parenchymatous changes in the kidneys and myocardium. The association of lesions most frequently encountered is that of subcutaneous hyperemia with moderately enlarged lymph nodes, enlarged, granular liver with necroses, and an enlarged dark spleen with rounded edges.

[3] Donaldson, H.: The Rat, 1924, p. 267. Data on spleen weights of albino rats.

Microscopic Examination of Organ Smears.—Since smears showing typical or atypical P. pestis may be considered the sixth cardinal sign of plague (Williams [4]), it appears worth while to discuss the microscopic findings as they were made at the time the cadavers were examined. Only the animals which were proven by subsequent tests to be infected with the hemorrhagic septicemia organism are considered.

The complete microscopic examination of the organ smears of 30 rats revealed coccoid bacilli, some bipolar in appearance, in the spleen (26 animals), the lung (19), the liver (14), lymph nodes, subcutaneous tissue, heart blood (3), and pleural effusion (2 animals). Four cadavers were definitely decomposed and the smears contained a considerable number of bacteria which were not characteristic.

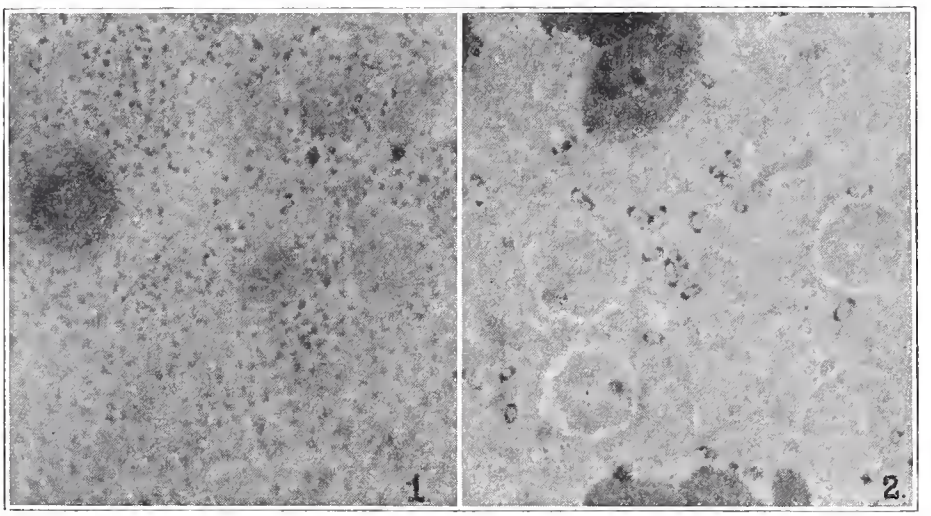

Fig. 1.—Pasteurella muricida, spleen smear, wild rat (1), and Pasteurella pestis, spleen smear, white rat (2). Both tinged with Wayson's stain x 1300.

Although small ringlike coccoid forms predominated and were quite often very numerous in the spleen and lung smears, the examiner was in the majority of instances unable to decide whether the bipolar organisms were atypical plague bacilli or typical hemorrhagic septicemia organism. However, smears prepared from the pneumonic area were nearly always characteristic, the microscopic field revealed an enormous number of small cocoid bacilli which gave a negative bipolar staining reaction but were surrounded by a fine capsule (fig. 1 [1]). When B pestis were present in large numbers in a microscopic field the diplococcoid, slightly elongated rods stood in definite contrast to the small coccoid pasteurella organisms (fig. 1 [2]).

The liver as a rule contained very few or no bacilli at all, unless small necrotic granules were dissected and crushed on slides. The same findings were made on the lungs without pneumonic lesions. Equally important is the fact that bipolar organisms were only demonstrated in the lymp nodes of 3 of the 30 rats carefully examined. The Indian Plague Commission [5] has shown that a bubo gives as a

[4] Am. J. Pub. Health, 1920, 10, p. 851.
[5] J. Hyg., 1907, 7, p. 338.

rule a better chance of finding plague bacilli than the spleen and the spleen than the heart blood. In rat pasteurellosis the predominant lesions are in the spleen and lungs and it is in these tissues that the microscopist must search for the bacteria.

Inoculation of Guinea-Pigs and Rats.—The foregoing pathologic and microscopic findings are suggestive of rodent plague. Wayson[1] has fully discussed some of the features which might be of assistance in establishing a differential diagnosis. A less experienced plague worker would probably in the majority of instances be unable to separate the many isolated lesions which resemble acute or subacute plague. He will always wish to confirm his suspicions by animal inoculations and cultures. The numerous difficulties which may be encountered have been analyzed in the first paper of this series.

In order to arrive at a definite diagnosis guinea-pigs and rats were inoculated with pieces of the diseased spleen, liver or lung tissues. The data which deserves consideration are summarized in tables 1 and 3.

TABLE 3

Tissue Inoculations of 62 Wild Rats

Tissues of 62 Suspected Rats	Hemorrhagic Septicemia	Guinea-Pigs		Rats		Remarks
		Pocket Method	Cutaneous Method	Pocket Method	Conjunctiva Method	
Spleen	Positive	21	2	12	1	1 plague; 1 hemorrhagic septicemia and plague; 1 hemorrhagic septicemia and B. enteritidis
	Negative	10	8	0	0	
Liver	Positive	13	2	6	—	1 hemorrhagic septicemia and cultures of B. pseudotuberculosis
	Negative	8	4	—	—	
Lung	Positive	5	—	—	1	
	Negative	1	—	—	—	
Total.....		57	16	18	2	

The postmortem findings of 70 rats suggested pasteurellosis, plague or rodent typhoid. One animal presented enlarged bronchial lymph nodes and cheesy pneumonic areas from which B. pestis was isolated on gentian violet plates. Seventeen rats exhibited no abnormalities. Of the 73 guinea-pigs inoculated either by the pocket or the cutaneous method with spleen, liver or lung tissues, 43 died in from 12 hours to 16 days. The necropsies showed typical lesions of acute hemorrhagic septicemia in all but three animals.

One guinea-pig had large buboes and other signs characteristic for plague; two others succumbed to the putrefactive anaerobes present in the original tissues. Since the cultures prepared on gentian violet agar

proved a coexistent infection of B. pestis, B. pseudotuberculosis and B. enteritidis with the pasteurellosis three guinea-pigs inoculated with liver tissues must be eliminated from consideration. Twenty one of the successfully inoculated 37 guinea-pigs were inoculated with spleen and 13 with liver specimens. Simultaneously the lung tissues of five rats were injected into a separate series of guinea-pigs, they became the victims of pasteurellosis in the same time as the animals infected with the splenic pulp. Rats inoculated with organs which were proven by guinea-pig inoculations to harbor the hemorrhagic septicemia organism died in from 12 to 36 hours. Even the application of organ juice from the spleen or liver on the conjunctiva resulted in fatal infections in from 24 to 48 hours. Attempts to produce pasteurellosis in eight white rats by feeding highly virulent tissues of wild rats were non-successful.

TABLE 4

MORTALITY OF GUINEA-PIGS AND RATS INOCULATED WITH TISSUES OF WILD RATS

	Number of Guinea-Pigs which Died Following the Infection by		Number of Rats which Died Following the Infection by	
Death in Hours	Subcutaneous Route	Cutaneous Route	Subcutaneous Route	Conjunctival Route
12	1	0	3	0
18	17	3	1	0
24	10	2	7	2
36	4	0	4	0
48	2	1	0	—
60	2	0	0	0

A diagnosis of an uncomplicated pasteurella infection in wild rats can be made rapidly. The data presented in table 4 make it sufficiently clear that a large percentage of the guinea-pigs and rats died acutely, that is, in from 12 to 60 hours. The highest percentage of death, 75%, occurred on the 18th to the 24th hours.

The acuteness of the inoculation disease is unquestionably due to the high virulence and the massive dose which was applied by the subcutaneous route. In guinea-pigs the introduction of the bacilli by vaccination did not delay the fatal issue for more than a few hours. White rats are slightly less susceptible than guinea-pigs; the subcutaneous introduction of tissues teeming with bipolar bacilli produced the greatest percentage of death between the 24th and 36th hour. These observations will be considered more carefully in connection with a discussion of pathogenicity experiments on tame and wild rats.

A further study of the data shown in tables 1 and 3 reveals, however, an important fact. The macroscopic and microscopic diagnosis of

pasteurellosis on 24 cadavers has not been confirmed by animal inoculations. In two cases the cultures proved the nature of the disease (hemorrhagic septicemia) but the guinea-pigs and the rats injected with the organs of the remaining 22 wild rats survived and the cultures from the inoculated animals were inconclusive or completely negative.

At first it was thought that a fifth disease perhaps due to a protozoan parasite was responsible for the suggestive lesions. Later it was realized that the cadavers had not been fresh and the inoculations of the laboratory animals were either made subcutaneously with very small pieces of the carefully flamed organs or by the cutaneous application of tissue particles on the shaved skin of the abdomen. The latter procedure was chosen to avoid secondary infections. Intentionally the surface of the shaved area was neither scarified nor were the organs rubbed into skin.

The well known fact[6] that the virulence of the hemorrhagic septicemia organisms may rapidly diminish in the organs of decomposed cadavers held at temperatures above 15 C. has been the subject of a recent study by Pfeiler and Kohlstock.[7] These workers recommend the intraperitoneal injection in rabbits and guinea-pigs since the infectiousness of the material containing bipolar organisms is exceedingly varied. If fresh, numerous virulent organisms may be present, sufficient to kill rabbits or guinea-pigs inoculated subcutaneously by the official method employed in Germany; if not fresh, negative results may be obtained. The intraperitoneal injection of rabbits is recommended even if the suspected material contains putrefactive organisms since these animals are resistant to them. Experiments with putrid tissues of infected laboratory rats have confirmed the observations of Pfeiler and Kohlstock and it is therefore not unlikely that the negative animal inoculations resulted from the use of tissues containing very few viable and virulent pasteurella organisms.

In future work it is recommended that as a confirmatory test for rats macroscopically diagnosed as hemorrhagic septicemia or for rats suspected of being pasteurella infected, at least two animals should be inoculated. The spleen pulp should be rubbed into a shaven area of the abdomen and a suspension of several organs should be injected intraperitoneally into a rabbit. However, the problem to recognize every case of pasteurellosis rarely enters into the routine diagnostic work of a rat survey. As soon as hemorrhagic septicemia as a confusing factor in the macroscopic diagnosis of rodent plague has once been established, every possible effort is naturally made to detect the cases of plague which might escape recognition. Special culture mediums and guinea-pigs immunized against hemorrhagic organisms may be absolutely necessary. However, in the light of the findings here recorded and in part

[6] Kitt: Kolle u. Wassermann, Handb. d. pathog. Mikroorganismen, 1913, 6, p. 49.
[7] Ztschr. f. Infektionskr. d. Haustier, 1921, 21, p. 276.

confirmed by laboratory tests it may be advisable to try the removal of the pasteurella organized by allowing the organs to putrefy before they are used for the cutaneous inoculation of the guinea-pigs. In the experience of this laboratory enrichment of the organs in 1/400% gentian violet broth for several days preceding the animal tests may eliminate the invasive bacilli and increase B. pestis.

In commenting on the nonsuccessful animal inoculations with tissues of wild rats which exhibited lesions considered characteristic for hemorrhagic septicemia another possibility should not be overlooked. The subcutaneous injection and the enlargement of the lymph nodes was very slight in one-half of the cadavers; pneumonic areas were only present in 4 (18%) of the total series of 22 rodents. The enlarged liver and spleen contained necroses in a relatively high percentage. Enrichment cultures in an improved brilliant green broth * were unfortunately not made and therefore the final proof is outstanding, but more recent observations on a large series of rats favor the view that at least 60% of the bacteriologically undiagnosed rat cadavers had the morbid lesions of rat typhoid due to either B. aertrycke or B. enteritidis.

Finally this large percentage of unconfirmed macroscopic diagnoses should furnish ample argument to those who wish to perfect the technic of the plague laboratory.

Diagnosis by Cultures.—At the beginning of this study plain hormone agar was used for the cultivation of the bipolar bacilli microscopically demonstrable in the spleen, liver and lung or exudate. Only five series of plates prepared with the tissues of 11 cadavers which were proven by animal inoculations to harbor the pasteurella organism were successful. Isolated colonies developed and could be picked for further identification. On six series of plates spreading contaminators submerged the small colonies of the bipolar bacilli. With the introduction of a special sodium sulphite, gentian violet agar,† the direct isolation of the hemorrhagic septicemia organism was accomplished from the tissues of 28 wild rats. The many advantages of this medium which have already been discussed, enable the plague worker to make a quick differential or confirmatory diagnosis. By increasing the concentration of the gentian violet to 1/500 or 1/400% the pasteurella and the most troublesome contaminations are as a rule inhibited and a coexistent plague infection may be recognized. The available evidence supports

* Sugar free veal broth 1,000 cc.; brilliant green (Grübler) 0.5 gm.; gentian violet 0.1 gm.

† Fresh 10% Na_2SO_3, 0.025 cc., and 1/750% genian violet (1.5 cc. of a 1: 1,000 solution per 100 cc. agar).

the contention that cultural methods on gentian violet agar are probably the most satisfactory when fairly fresh cadavers of wild rats are available. In this laboratory the organs are usually cultured first, in the meantime the tissues are held on ice and animal inoculations are only made when the plates are unsatisfactory or show colonies of interesting micro-organisms.

Bacteriology of the Causative Organism.—Thirty-four strains isolated by direct plating or by animal passage have been subjected to a comparative study.

Morphology: The organism examined directly from animal tissues is a small bacillus, which may or may not give the bipolar staining reaction. It is frequently coccoid in shape and may be mistaken for a pneumococcus (Giemsa's or Wayson's stain). It measures 0.3 to 0.75 μ in length and 0.2 μ in width and not infrequently shows a definite capsule. The microbes in the films from the exudate, tissues and cultures tend to stain solidly; the longer forms may exhibit bipolar staining but rarely in the manner typical for B. pestis (fig. 1). It takes the counterstain in Gram's method. Aggregations in form of clusters or short chains (3 to 5 individuals) are sometimes noted in smears prepared from pneumonic areas.

The morphology of the bacterium varies with the time which has elapsed since its isolation and the medium on which it is cultivated. Recently recovered, virulent strains maintained on blood mediums at 30 C. for successive passages show minute, coccoid, immotile rods. Cultivation on meat extract agar produces longer, pleomorphic rods, at least one half the size of B. pestis. In the water of condensation, in some of the small, retarded colonies which are noted on plain agar plates and in mediums of unfavorable reaction or composition (3% salt agar) involution forms are most marked. However, the pleomorphism is less striking than that ordinarily exhibited by the plague bacillus.

Cultural Characteristics: All strains are strongly aerophilic. When first isolated from spontaneous infections they grow very feebly or not at all on ordinary mediums without the addition of sulphite or blood extracts (hormone mediums). Heavy inoculums are necessary to perpetuate the strains and repeated subcultures are required to maintain the viability of the cultures. After several transplants on blood or hormone agar good and usually confluent growth occurs on ordinary solid mediums, provided that a sufficient quantity of material is carried over.

Agar: On hormone agar (P_H 7.4) at 30 C. the surface colonies of recently isolated strains are circular in shape, small dewdrop-like with even borders. They are whitish with a knob-like opaque center and more transparent toward the edges. Stock cultures form large (2 mm. diameter) grayish, opaque, coherent colonies. Two types of surface colonies are noted on meat extract agar plates—large grayish and minute irregular granular droplet-like discs. The latter are dwarf colonies and have nothing in common with the smooth and rough disassociations frequently encountered in P. leptisepticum cultures. They may reach a diameter of ½ mm. and they are just as virulent as the large types.

On certain batches of meat extract but very rarely on hormone agar the colonies are viscid, adhere to the mediums, they may be removed en bloc. A similar observation has been recorded by Kister and Schmidt[8] for the ferret

[8] Centralbl. f. Bakteriol., I, O., 1904, 36, p. 454.

strains of hemorrhagic septicemia. On slanted agar the growth may form a diffuse, thin, grayish, somewhat stringy film.

Blood Agar: Large, whitish, nonhemolytic colonies develop rapidly at 30 to 37 C.

Gelatine: Delicate, smooth and round grayish colonies develop very slowly on plain gelatine somewhat more rapidly on hormone gelatine plates. The medium is never liquefied From a diagnostic point of view it is noteworthy that the pasteurella organism fails to grow in gelatine pour plates held at 4 C. while P. pestis regularly develops into typical yellow-grayish, granular pin point colonies. In stab cultures the growth is confined to the track in the form of small pearly colonies.

Broth: The growth depends entirely on the composition of the medium. It may be in form of a diffuse turbidity or it may be granular. In some preparations a definite pellicle or ring of vegetation with excellent stalactite formation or a flocculent, occasionally slimy, coarse sediment may form in the gradually clearing liquid. Even small flakes may appear along the wall of the tube. Such a culture cannot be distinguished from that of P. pestis. Old cultures have always a very characteristic bitter almond-like odor, which is probably due to volatile metabolic products containing sulphur. The reaction becomes slightly alkaline (from P_H 7.0 to 7.8). The addition of blood or blood serum enhances the growth.

	Rat pasteurella (34 Strains)	P. pestis (18 Strains)	B. pseudotuberculosis (4 Strains)
Glucose	Acid	Acid	Acid
Levulose	Acid	Acid	Acid
Galactose	Acid	Acid	Acid
Sucrose	Acid	No change	No change
Maltose	No change (3 old strains acid)	Acid	Acid
Lactose	No change	No change	No change
Mannitol	Acid	Acid	Acid
Dulcitol	No change	No change	No change

However, frequent transplantations are necessary in order to keep the cultures alive. Glucose has no influence on the growth vigor

Potato: The growth is only observed when the reaction of the potato is changed by the addition of sodium carbonate. Tyrode solution with small pieces of sterile raw potatoes gives excellent growth.

Peptone Solution: Heavy seedings are required to start a culture and a scanty but diffuse growth develops.

Bromcresol Purple Milk: The milk remains either unchanged or an increase of the hydrogen-ion concentration from P_H 6.8 to 6.6, but no coagulation is noted.

Salt Agar: On 3% sodium chloride agar the growth is very scanty, mostly composed of diplococcoid and bacillary forms.

Biochemical Reactions: Since several strains of the rat organism gave the reactions of the hemorrhagic septicemia group, only eight carbohydrates in which variations have been reported were used in a study of the fermentation reactions of the entire collection.

Some difficulties were encountered in making a selection of the most suitable basic medium. Brom-cresol purple or Andrade's decolorized fuchsin served as indicators. Meat extract broth (P_H 7.0; buffer index (BI),[9] 2.14), meat extract, semisolid agar (P_H 7.0; BI, 1.74), meat extract, semisolid agar with 5% rabbit or beef serum (P_H 7.0; BI, 1.74), peptone solution (P_H 7.0; BI, 1.36), peptone solution with 5% rabbit serum (P_H 7.0; BI, 1.36), Enlow's monobasic potassium phosphate, peptone agar (Enlow's medium 1)[10] (P_H 7.0; BI, 4.44),

[9] Brown: J. Bact., 1922, 6, p. 555.

[10] Pub. Health Rep., 1923, 38, pp. 2129, 2140.

Enlow's monobasic sodium and dibasic potassium phosphate, peptone agar, (Enlow's medium 2)[10] (P_H 7.0; BI, 6.98), Enlow's 1, with 5% serum (P_H 7.0; BI 4.44), Enlow 2 2 with serum (P_H 7.0; BI, 4.44), Hiss' serum water (P_H 7.0; BI, 2.8), Hiss' serum semi-solid agar (P_H 7.0; BI 3.0) and sugar free veal infusion (P_H 7.0; BI 5.0) with 0.5% carbohydrate were tried in a comparative series.

Thirty-four strains of the rat pasteurella, 18 strains of P. pestis and four strains of B. pseudotuberculosis were repeatedly subjected to the tests. With the exception of Enlow's mediums 1 and 2 the reactions were constantly the same and strikingly uniform for all strains.

The final hydrogen-ion concentration in 1.0% glucose, saccharose and mannitol in peptone serum or Hiss serum water incubated at 30 C. for 5 days varied between P_H 5.8 to 6.2. The reaction of the lactose or of the maltose medium seeded with recently isolated strains remained at the initial hydrogen-ion concentration or advanced from P_H 7.0 to 7.4 on prolonged incubation. Old strains repeatedly acquired the property to ferment maltose. The ability to ferment saccharose has remained constant and this property differentiates the rat pasteurella from the true P. pestis which does not hydrolyze this carbohydrate.

TABLE 5

FERMENTATION REACTIONS IN DIFFERENT MEDIUMS AFTER FIVE DAYS INCUBATION AT 30 C.

	Peptone Solution BI 1.76* P_H 7.0		Peptone Solution with Serum BI 1.36 P_H 7.0	Hiss' Serum Water BI 2.8 P_H 7.0		Enlow's Medium 1 BI 4.4 P_H 7.0		Enlow's Medium 2 BI 6.98 P_H 7.0
Carbohydrate	P. muricida	P. pestis	P. muricida	P. muricida	P. pestis	P. muricida	P. pestis	P. muricida
Glucose	6.2	4.8	6.2	6.3	5.0	7.0	5.8	7.0
Saccharose	6.3	6.6	6.4	6.2	6.8	7.0	7.0	7.0
Maltose	7.0 (6.4)	5.2	7.0	7.0	5.2	7.0	6.0	7.0
Lactose	7.4	7.0	7.2	7.2	6.8	7.0	7.0	7.0

* Buffer index.

The selection of a basic medium is most essential in order to obtain conclusive fermentation reactions. In this laboratory the routine carbohydrate tests are usually made in Enlow's medium 1 or 2. When six rat pasteurella strains were compared with three P. pestis strains, striking differences were observed. The glucose, levulose and maltose tubes seeded with the plague bacilli indicated by the change of the dyes (Andrade's or bromcresol purple indicator) fermentation of the sugars and acid production. The same test mediums inoculated with the rat pasteurella revealed growth but the hydrogen-ion concentration remained constant even on prolonged incubation. At first these discrepancies were attributed to an absence of growth accessory factors in the Enlow's medium, however, the addition of serum or sterile potato extract enhanced the growth but failed to change the P_H. Furthermore, parallel tests run in peptone, Hiss' serum water and semisolid meat extract carbohydrate mediums gave reactions which harmonized fully with the data published by Magnusson,[11] Jones,[12] d'Aunoy,[13] Besemer,[14] Pons,[15] and others. The data summarized in table 5 offer an explanation. The buffer index of the two Enlow's mediums is very high (BI 4.44 and 6.98) and it is reasonable to suspect that the small amount of lactic acid formed in the course of the fermentation of the glucose or saccharose is constantly

[11] Ztschr. f. Infektionskr. d. Haustier., 1914, 15, p. 21.
[12] J. Exper. Med., 1921, 34, p. 561.
[13] J Infect. Dis., 1923, 33, p. 391.
[14] J. Bact., 1917, 2, p. 177.
[15] Ann. de l'Inst. Pasteur, 1925, 39, p. 884

buffered by the phosphates. P. pestis (final P_H 4.8 to 5.2) produces more acid and thus overcomes the high reserve alkalinity. It is not claimed that the buffer content is the only factor which determines the amount of demonstrable acid formed by the hemorrhagic septicemia organisms. Recently isolated body strains grow poorly on mediums which lack certain biocatalysts present in the serum. Since these cultures require immediate identification it was found advantageous to use peptone serum mediums with a low buffer index of 1.36. Hiss' serum water is equally satisfactory as a basic medium although the index is slightly higher (BI 2.8). It is needless to emphasize that the serum should be tested for glucose and a control tube with a delicate indicator but no carbohydrates should be included in the series.

Indol and Reduction of Nitrates: Every strain produced indol in tryptic blood digest broth and reduced nitrates to nitrites.

Bile Solubility: All strains are insoluble in ox and guinea-pig bile.

Growth Accessory Factors: The pasteurella organism grows best and retains its virulence in a nutrient broth containing a small amount of raw or cooked blood. These observations recalled the recent studies[16] on the X and Y factors. A series of experiments have been undertaken to determine the nature of the active substance of the blood.

Thus it was found that blood agar shaken with charcoal to absorb the X factor favored the development of large colonies while autoclaved blood agar without the addition of sodium sulphite when seeded lightly remained sterile or gave rise to very small colonies. Filtered potato extract agar heavily seeded gave no growth. However, raw potato particles in tyrode solution furnished excellent and highly virulent cultures.

The details of these experiments will be reported in a separate paper in which the recent observations of Webster and Baudisch[17] on the biocatalytic activity of iron compounds in the growth of B. lepisepticum will be correlated with that of sodium sulphite.

Type Dissociation in Old Broth Cultures.—In the course of some experiments a six months old hormone broth culture was smeared on plain agar. The growth consisted of large, thick, yellowish, iridescent coli-like colonies which differed from the types isolated directly from the rats.

Microscopically the bacilli resembled very closely the pasteurella organism although they were slightly larger and still possessed a capsule (Cyanochin and Burri method). The individual colonies were round, distinctly raised, quite opaque, with an iridescent, slightly yellowish tinge and smooth edges. Held at room temperature the color deepened to a light brown and some colonies showed a knoblike center. When touched with the needle they appeared mucoid but not stringy. On agar slants the 24 hours old growth was blue grayish iridescent; the water of condensation had a granular sediment. The characteristic odor of the pasteurella culture was missing. In plain broth a slight turbidity was present during the first 24 hours. After a few days' standing at 30 C. the medium became clear and a fine slimy sediment developed which was readily resuspended. The fermentation reactions were identical with those of the original body strains with the exception of mannite which was not attacked. The broth cultures were not cleared by the addition of bile. A polyvalent antiserum prepared with virulent

[16] Knorr: Ergebn. d. Hyg. Bakteriol. Immunitsf. u. exper. Therap., 1925, 7, p. 641. Complete Summary.

[17] J. Exper. Med., 1925, 42, p. 473.

strains failed to agglutinate the avirulent culture. Killed cultures were toxic. Guinea-pigs, rats, rabbits and pigeons tolerated the subcutaneous injection of 1 cc. of broth or 2 loopfuls of agar slant cultures.

The avirulent rat pasteurella variants have been used in immunization experiments which will be described in the third paper of this series. This accidental recovery of an avirulent variant which corresponds in every detail with that isolated and described by Manninger [18] from an old broth culture of P. avisepticum recalled the observation of de Kruif,[19] Webster [20] and others. Numerous attempts were made to demonstrate the existence of virulent and nonvirulent variants in 72 hours old broth cultures held at 37 C. in shallow layers. In some experiments attenuation was tried in alcoholic broth according to the observations of Hetsch.[21] Granular colonies which grew in form of a coarse sediment in plain broth were sometimes recovered but they proved just as virulent as the original smooth cultures. From the work completed it is thus evident that prolonged cultivation in plain meat extract broth is essential in order to recognize by the customary methods a disassociation of the highly pathogenic rat pasteurella strain into virulent and avirulent variants. This conclusion reached after several months of intensive study was a great disappointment since the plague worker who had to deal with coexistent pasteurella infections in wild rats would have welcomed the opportunity to isolate rapidly an avirulent strain suitable for quick immunization of guinea-pigs. On the other hand the observations may have greater significance. The rat cultures are epidemiologic strains probably much more resistant to modifying influences than those species of the hemorrhagic septicemia group which yield under suitable conditions a virulent and avirulent variant. The biological or biochemical factors responsible for these differences are now the subject of further study.

Serologic Reactions.—Ten strains of the pasteurella were inagglutinable in the antiplague serums secured from the Pasteur and the Swiss Serum Institut or prepared in this laboratory (titer of 1 : 3,000).

As antigens formalinized suspensions were used, 18 hours old hormone agar cultures in large Blake bottles were washed off with 5 to 8 cc. of a 0.1% formalinized salt solution. It is most important that the formalin is added to the physiological salt solution and not to the bacterial suspension which has been washed off. The dense preparations were kept at room temperature and tested

[18] Centralbl. f. Bakteriol. I, O., 1919, 83, p. 520.
[19] J. Exper. Med., 1922, 35, pp. 561, 621.
[20] Ibid., 1924, 39, pp. 837, 879; 40, p. 117; 41, p. 571.
[21] Ztschr. f. Hyg. u. Infectionskrankh., 1904, 48, p. 442.

for sterility at regular intervals. As a rule the bacilli were killed on the 2nd to the 3rd day. A homogeneous, stable suspension was prepared in formalinized salt solution and standardized nephelometrically when needed. The agglutination reactions with such antigens are flaky and very easily read when the tubes are incubated at 55 C. for two hours. The clumping begins almost at once and complete sedimentation is reached within three hours.

The stock suspension was used for the immunization of several rabbits. Four to five injections (0.5 cc. 1st day subcutaneously; 1.0 cc. intravenously 4th and 11th days; 2 cc. intravenously on the 17th and 25th days—test bleeding 30th or 35th day) produced serums with stable titers of 1:2560 to 1:6000. Thirty-two strains and one strain of Pasteurella avisepticum were agglutinated by these serums to the titer limit. Two strains were partially clumped in dilutions varying from 1:320 to 1:640. Sixteen strains of P. pestis were not sedimented in dilutions higher than 1:10. Normal serum agglutinated the hemorrhagic septicemia and the plague strains in this dilution.

These observations leave no doubt that the bacilli isolated from the rats represent an immunologically uniform group. They furthermore lend support to the belief that an epidemic strain of the pasteurella group was responsible for the rodent disease.

The methods of producing diagnostic antiserums for P. pestis and the pasteurella group have been carefully studied and although the observations will be the subject of a separate paper it must be noted that heated or carbolized bacterial suspensions are poor antigens, which are frequently highly toxic. Even living organisms require frequent and prolonged injections to yield serums with a titer of 1:500. Since intensive immunization tends to reduce group specificity it is reasonable to suspect that the failure of several workers (Tanaka [22]) to demonstrate immunologic subgroups or types must be ascribed to the production of the antiserums.

The agglutination tests afford a rapid and efficient method for the differentiation of P. pestis from members of the hemorrhagic septicemia group which attack wild rats.

Experimental Infections and Virulence.—The bacteriologic studies are sufficiently definite to diagnose the causative organism of the wild rat disease. However, for reasons to be outlined, it appeared of interest to determine the virulence of the bacilli for various species of laboratory animals. It is generally recognized that the virulence of the representative of the hemorrhagic septicema group for different animals may vary considerably. As a rule it is greatest for those species which are prone to spontaneous infections. It may, however, be enormously increased by animal passage but the modified strain invariably exerts a more

[22] J. Infect. Dis., 1926, 38, p. 421.

violent action on the species of animals from which it has originally been isolated. The property of being highly virulent when inoculated in body fluids such as blood or exudates is common to all variants. Continuous cultivation on mediums which contain blood preserve the virulence (Iyengar [23]) while transfers through mediums lacking certain biocatalysts may render the bacilli avirulent. Such cultures rarely regain their pathogenicity by animal passage.

An excellent opportunity was afforded to verify these conceptions. If the variant of the hemorrhagic septicemia bacillus is specific then the strains should be highly pathogenic for rats since Lignières [24] has shown that the pasteurella organism isolated from birds, cattle, sheep, hogs, etc., are nonvirulent for this species of rodent on subcutaneous inoculation, (0.25 cc. peritoneal exudate from an infected guinea-pig). Massive doses of cultures and body fluids inoculated intraperitoneally may be fatal. Neumann [25] who studied a representative of the bipolaris group isolated from a wild rat was unable to infect these rodents by intraperitoneal, subcutaneous or conjunctival route. However, he reports that he succeeded in producing the disease by inhalation or feeding of the bacterium. On the other hand the pasteurella strain isolated by Kister and Schmidt [8] in the course of an epidemic among ferrets which had experimentally been used for the suppression of rats along the harbor terminals of Hamburg was highly pathogenic for tame rats. The organism accidentally recovered by Smillie [26] in the course of his search for leptospira in the kidneys of wild rats near São Paulo was nonpathogenic for these rodents on intraperitoneal injection. Gheorghin [27] isolated from crows a pasteurella which was highly pathogenic for rats. These reports indicate that a number of variants of the bipolaris group may cause infections or exist as saprophytes in the tissues of wild rats. Aside from furnishing confirmatory evidence to some of the published findings it was anticipated that the experimental reproduction of the disease would throw some light on the mode of transmission and the epidemiology of the rat disease.

The data dealing with the numerous tests are summarized in table 6. In adopting the terminology of Rowland [28] it is deemed advisable to designate in the experiments the origin of the infective material as body, serum or culture strains.

Inoculation of Guinea-Pigs: The experiments with subcutaneous injections of tissues of spontaneously infected rats have already been recorded. Inoculations with exudates or organs from other animals (guinea-pigs, rats, mice) and with cultures act in a similar manner. As a rule 0.1 cc. (approximately 40 billion organisms) or a culture in hormone broth, 2nd and 3rd generation after a direct isolation from the rat body produces a fatal infection in 9 to 24 hours. The symptoms and necropsy findings are best illustrated by the history of one animal.

[23] Indian J. M. Research, 1926, 13, p. 555.
[24] Contribution a l'étude des Septicémies hémorragiques, 1900.
[25] Ztschr. f. Hyg. u. Infektionskr., 1903, 45, p. 450.
[26] J. Infect. Dis., 1920, 27, p. 378.
[27] Compt. rend. Soc. de biol. 1922, 87, p. 285.
[28] J. Hyg., Plague Supplement 4, 1915, p. 765.

Male guinea pig, weigbt 295 gm., inoculated subcutaneously on abdomen with 0.1 cc. of an 18 hours old hormone broth culture,10tb generation; died in 16 hours.

Necropsy findings: The site of the inoculation is indurated and shows in the subcutaneous tissue a sanguinolent, gelatinous edema extending over an area the size of a dollar, the blood vessels are deeply injected and ooze dark, tarry blood; the muscles are injected and brick red discolored, particularly in the inguinal and axillary regions; the axillary, submaxillary and inguinal lymph nodes are sligbtly enlarged and red; the peritoneal cavity contains 4 cc. of blood tinged flaky exudate; a fine layer of fibrin covers the liver, the spleen and the deeply injected intestinal loops, which contain a bile stained chyme; the omentum shows small hemorrhages; the liver is dark in color and friable; the spleen is not enlarged but the edges are rounded; tbe color is purplish and on section the pulp is dark brown and very soft; the

TABLE 6

PATHOGENICITY OF PASTEURELLA MURICIDA (N. SP.). METHOD OF INOCULATION

Animal		Strain and Amount of Infective Material Inoculated: Method	Strain	Amount	Number of Animals	Time of Death, Hours
Guinea-pigs	a	Subcutaneous	Body	Variable	48	12 to 60
	b	Subcutaneous	Culture	0.1 cc.	34	9 to 24
	c	Cutaneous	Body	— spleen	25	24 to 144
	d	Cutaneous	Culture	1 loopful	9	24 to 168
	e	Peritoneal	Culture	0.001 cc.	5	12 to 18
	f	Nasal	Body	Spleen	4 (2 positive)	36 to 48
	g	Conjunctival	Body	Spleen, liver and lung	8	Negative
Rabbits	h	Subcutaneous	Culture	0.1 cc.	19	12 to 24
	j	Intravenous	Culture	0.01 cc.	1	8
	k	Intrapleural	Culture	0.1 cc.	4	6 to 12
Mice	l	Subcutaneous	Culture	0.1 cc.	6	40 to 48
White rats	m	Subcutaneous	Body	Spleen	14	12 to 36
	n	Subcutaneous	Old cultures	0.1 cc.	5	72 to 120
	o	Cutaneous	Culture	1 loopful	2	120
	p	Intraperitoneal	Culture	0.1 cc.	5	72 to 120
	q	Intrapleural	Culture	0.1 cc.	2	16
	r	Nasal	Culture	1 drop	4 (2 positive) epidemic	96
	s	Conjunctival	Body	Spleen	6	24 to 48
	t	Feeding	Body	Spleen and liver of guinea-pigs and rats	18	Negative
Wild norvegicus rats (San Francisco and Los Angeles)	u	Subcutaneous	Culture	0.1 cc.	9	12 to 16
	v	Subcutaneous	Old cultures	0.1 cc.	4	96 to 120
	w	Cutaneous	Body	Pleural fluid	2	120
	x	Intraperitoneal	Old culture	0.1 cc.	5	12 to 120
	y	Intrapleural	Body	Drop of pleural	1	18
	z	Nasal and conjunctival	Body	Spleen	2	72
	aa	Nasal and conjunctival	Culture (recent)	1 drop	6	18 (plague coexistent) to 168
	bb	Nasal and conjunctival	Culture (old)	1 drop	2	120
	cc	Feeding	Body	Cadavers	10	No symptoms
Pigeons	dd	Intramuscularly	Body	1.0 cc.	2	No symptoms
Chickens	ee	Intramuscularly	Body	1.0 cc.	1	No symptoms
Cat	ff	Subcutaneously	Body	1.0 cc.	1	No symptoms

pericardial sac is distended by a turbid, stringy, fibrinous exudate; flakes of the exudate are also noted on tbe deeply congested partially callapsed lobes of the lungs; from both pleural cavities 1.5 cc. of exudate are collected. Microscopically and culturally the tissues contain the sbort coccoid bacilli.

The organism has retained a maximum virulence on continuous artificial cultivation. Successively transferred every two weeks through serum broth proved highly virulent for guinea-pigs at the end of 16 weeks. Approximately 50 organisms subcutaneously injected killed a guinea pig weighing 700 gm. in 72 hours. It is therefore not unlikely that the smallest possible dose (1 to 10) of virulent

bacilli present in body fluids of rats may fatally infect the highly susceptible guinea-pig.

Intraperitoneal injections produced the same lesions with the exception that the amount of hemorrhagic, fibrinous exudate both in the pleural and peritoneal cavities frequently exceeded 10 cubic centimeters. Furthermore, an inoculum of 0.01 cc. of broth may cause a fatal infection in 12 hours.

The careful cutaneous application of one loopful of broth culture of a recently isolated strain on the shaven but not scarified skin produced the disease which caused the death of the guinea-pigs in 24 to 60 hours. The animals usually show on the 12th hour apathy and somnolence and a brownish crusty exudate surrounds the partially closed eyes. The vaccinated area is rarely indurated and the subcutaneous edema and necrosis present in the subcutaneously inoculated guinea-pig has not been noted. Aside from the abundant fibrinous exudate on the serous membranes, infarcts in the myocardium and extensive hyperemia of the tissues have been recorded. Strains which through continuous cultivation have lost their great virulence may infect by the cutaneous route when the skin is scarified. The post mortem findings resemble those of guinea-pigs infected subcutaneously. Since death is frequently delayed for five to six days the fibrin layers in the pericardium, on the liver, etc., are massive and necroses and abscesses are not uncommon in the lungs, spleen and liver.

Small particles or suspensions of infected rat spleens were placed or dropped into the nares without causing abrasions of the mucous membranes. Two of four trials led to a successful reproduction of the disease. The lesions were the same as already described. The instillation of crushed spleen or liver tissues into both conjunctival sacs of eight guinea-pigs failed to infect the animals. Although special experiments were not undertaken dead guinea-pigs with a septicemic distribution of the bacilli were often gnawed on by the mates placed as controls into the same cages without showing any ill effect.

Summary: The coccoid organism is highly virulent for guinea-pigs which can successfully be infected by the subcutaneous and peritoneal route. Recently isolated strains are pathogenic when placed on the uninjured mucous membrane of the nares. The lesions are constant and are indistinguishable from those produced by the representatives of the hemorrhagic septicemia group. For a period of 16 months the strains cultivated on hormone agar have maintained their virulence and kill regularly when injected subcutaneously in amounts varying from 10^{-1} to 10^{-10} of an 18 hours old broth culture.

Rabbits: Infections of rabbits weighing 2,500 to 3,000 Gm. are readily secured by intravenous, subcutaneous or intraperitoneal injections of young cultures. The bacilli are highly virulent for these animals since an intravenous injection of 0.01 cc. of a culture may kill in eight hours producing all the characteristics of hemorrhagic septicemia; general injection of the blood vessels, hyperemia of the liver and lungs, splenic tumor, extensive serofibrinous pericarditis and pleurisy, and hemorrhagic tracheitis.

Mice: Mice are less susceptible to the bacilli; 0.1 cc. of a broth culture inoculated subcutaneously killed in 40 to 48 hours. At necropsy a diffuse gelatinous edema with deep injection of the muscles, fibrinous exudation on the serous membranes, enlargement of the spleen and pulmonary hyperemia are constantly present.

Barn Yard Birds and Cats: Pigeons, chickens and cats are nonsusceptible to intramuscular or subcutaneous injections of 1.0 cc. of broth cultures.

White Rats: Body or blood broth strains of the pasteurella produce death regularly in 12 to 36 hours. Culture strains are less virulent and the fatal issue of the infection may be delayed for 72 to 120 hours. The pathologic picture is illustrated by the following history.

Rat, male, weight 215 gm. inoculated subcutaneously with 0.1 cc. of an 18 hours old broth culture (3rd generation maintained on hormone blood agar). Found listless with crusty exudate around eye on 3rd day, died on the 5th day.

Necropsy Findings: A slight cutaneous induration is found at the site of the inoculation; an extensive gelatinous, whitish edema extends along the abdominal and the thoracic regions, while a distinct injection of the blood vessels with hemorrhages is present near the axillary, inguinal and submaxillary lymph nodes which are enlarged, hyperemic and soft. Three cc. of a cloudy pus-like exudate is collected from the peritoneal cavity. The liver is dark red, slightly enlarged; the spleen is twice its normal size, the edges are rounded, the color is deep blue, reddish and the pulp is friable; a few drops of a straw colored exudate is found in both the pleural and pericardial cavities; the lobes of the lung are slightly congested and voluminous. The injected organisms are microscopically and culturally present in enormous numbers in the exudate and spleen. They are relatively scarce in the liver, heart blood and lungs.

A loopful of a recent broth culture rubbed in the shaven and slightly scarified skin, intrapleural or intraperitoneal injections regularly killed in 16 to 120 hours. The liver of two rats infected intraperitoneally and one by vaccination showed definite stippling due to numerous small necroses. The pleural effusions of the same animals consisted of 5 cc. of a serosanguineous exudate.

Two of four rats died following the introduction of a drop of culture into the nasal passages as follows.

The animals, etherized and an equal number unetherized, were treated by holding the head backwards. A drop of culture discharged from a medicine dropper was placed on the nares; the fluid was rapidly aspirated by the deep inspirations of the narcotized rat.

The instillation of body strains (pulped tissues) into the conjunctival sac is regularly followed by fatal infections. The following necropsy findings have been recorded:

Slight subcutaneous injection and enlargement of the inguinal and the axillary lymph nodes; gelatinous edema and hemorrhages in the submaxillary region; thorax distended by 3 to 8 cc. of straw colored effusion which is poor in fibrin; lung lobes grayish and completely collapsed, splenic tumor, dark and friable liver.

The feeding of highly infectious rat and guinea-pig tissues for periods extending over several days produced no illness. The rats (10) which were not affected by feeding proved susceptible by subcutaneous infection.

From the foregoing observations it may be concluded that the coccoid bacilli are highly virulent for white rats and although the susceptibility of these rodents is less than that of guinea-pigs, infections through the unbroken conjunctival and nasal mucous membranes may be secured when body or recently isolated blood agar strains are used. A spontaneous laboratory epidemic which will be described below, fully supports this statement.

Gray Norvegicus Rats: While the virulence of the bacilli for gray rats is proven by the experiments detailed in table 6, the susceptibility varies with different lots of these rodents. For example, the rats secured from the vicinity of Los Angeles succumbed in a shorter time than those originally caught in San Francisco. In fact, animals may be encountered which survive inoculations of old cultures unless given in large doses. However, subacute cases of the disease by way of the nasal or conjunctival mucous membranes are readily reproduced. The history of such an observation is herewith detailed.

Norvegicus rat 3, weight 300 gm. infected by nasal route with one drop of an 18 hours old culture, strain 36. The animal is active on the 2nd, 3rd and 4th day. On the 5th it shows labored respiration and general apathy. It is found dead on the 7th day. The necropsy notes record slight injection of the subcutaneous tissues, enlarged lymph nodes, dark stippled liver, enlarged dark spleen, deeply congested lung with numerous bronchopneumonic areas in the anterior lobes and grayish foci in the principal lobes. The lesions are indistinguishable from those seen in the wild rats which have been dissected in the course of the plague survey.

The resistance of the rodents may be lowered by other infections or by transferring the animals into a cold room. These conclusions are fully in harmony

with the present conceptions of the pathogenesis of the animal diseases caused by bipolar organisms. In order to test the efficacy of several methods which are used in this laboratory to recognize in plague rats the coexistence of the coccoid bacillus, a series of wild rats were given antiplague serum followed by a vaccination with plague tissues. Five to six days subsequently, a loopful of a pasteurella culture was smeared on the nose. Invariably the rats died overnight. The findings were those of acute hemorrhagic septicemia due to the secondary inoculation with the coccoid bacilli. Small necrotic foci were always present in the liver and in the spleen. The small bacilli predominate but plague bacilli may occasionally be seen in the smears. However, on gentian violet (1/400%) agar plates a pure culture of P. pestis may be obtained. There is no doubt that the plague infection increased the susceptibility. Since the transmission experiments by way of the nasal and conjunctival membranes were uniformly successful and since the viscera of rats which died of pasteurellosis were eaten on several occasions by the cage mates without causing any noticeable symptoms, separate feeding tests with cultures have not been carried out. The pasteurella strains here described differ from those investigated by Neumann[25] and Kister and Schmidt.[8] These workers have noted that the feeding of cadavers and sometimes of cultures killed rats in six to eight days. On the other hand, the great resistance of laboratory animals towards artificial feeding infections even with body strains of the hemorrhagic septicemia group are well known and need little comment. Chickens, for example, may ingest with impunity organ material which is highly virulent by the cutaneous or subcutaneous route.

Cultures kept for several months on plain agar lose their pathogenicity for white and gray rats. However, it can be restored by passage through guinea-pigs. A strain exalted for this rodent may again infect rats through the unbroken respiratory or conjunctival mucosa.

Spontaneous Laboratory Epidemic among White and Gray Rats.—The great affinity of the coccoid bacilli for the serous membranes of the thoracic cavity and for the pulmonary tissues, the successful reproduction of the disease by placing cultures on the mucosa of the nose and eye, the great resistance against infectious feedings supported the conception that the malady is contracted and spread among wild rats like a respiratory disease. Although the exact pathogenesis and the exact portal of entry, either the lymphatics of the eyes, the upper respiratory tract or the peri- and endobronchial tissues has not been studied. A spontaneous epidemic among the stock rats of this laboratory which took the form of a complete experiment proves definitely the dissemination of the pasteurella virus through discharges of the mouth and nose. The history of this epidemic is herewith briefly given.

In the course of certain immunity experiments a number of rat pasteurella strains were, by subcutaneous inoculations, passed in three series through white rats.

On April 9th the pleural exudate of the 3rd passage rat was smeared on the nares of two and the conjunctiva of one white rat.

On April 10th one of the inoculated rats was missing from the cage but was found in a corner of the isolation room. The animal, which appeared sick, was replaced in its cage.

On April 11th 54 white rats distributed in four large wire cages were found dead; two were dying; two white and one gray rat were ill but recovered. Necropsies, smears and cultures confirmed the diagnosis of hemorrhagic septicemia. The pleural cavities were completely filled with a light colored exudate which contained the coccoid bacilli in abundance. The lungs were collapsed and the spleens enlarged. The lesions corresponded closely to those observed in rats which succumbed in a peracute manner to a conjunctival infection. The rat which had been at large on the 10th of April was dead and presented the same post mortem findings. Twelve white rats in a separate cage, 14 guinea-pigs, 2 rabbits, 4 gray Norvegicus rats (from San Francisco) held in the same room remained well.

The blood of the recovered white (2) rat was tested for blood agglutinines with negative results and cultures of the respiratory tree were found sterile. Three white rats succumbed subsequently to pasteurellosis when exudates and splenic tissues were smeared on the nares of the 12 inmates of the cage in which no fatalities had occurred. Four of the surviving nine rats proved susceptible to the coccoid bacilli by subcutaneous injection. The immunity of the five remaining rats has not been tested. The four gray rats survived repeated trials to infect them by smearing the mouth, nose and eyes with virulent tissues. From previous experiments it was known that guinea-pigs and rabbits are highly resistant to rat passage strains applied to the nasal or conjunctival mucous membranes.

It is naturally impossible to reconstruct all the factors but the following succession of events led, in probability, to the epidemic among 73 rats isolated in an animal room.

The pasteurella strains were greatly increased in virulence by rat passages. The animal, which was at large for 12 hours, carried the infectious material on the nose, eyes and paws, and transmitted it to the inmates of at least seven cages. It is well known that tame white rats at large and climbing over the wire doors of the other cages stick their noses through the meshes. The rats in the cage jump up and lick or sniffle the head and the paws of the fore legs. One cage in a remote corner of the small isolation room, particularly reserved for plague work, was either not visited or, as subsequent tests indicated, the rats of this lot were very resistant to conjunctival or nasal infections. The Norway variety probably exposed, came from a group of San Francisco rats, which proved invariably far more resistant to pasteurellosis than the animals caught in or around Los Angeles where hemorrhagic septicemia infections have not been found. The three old rats (1 gray and 2 white) which survived a severe spontaneous infection never regained their original vitality; they had nasal discharges and moist bronchial rales. When sacrificed B. bronchisepticus and various cocci were isolated from the bronchii and from several unresolved pneumonic areas.

It is clear from the evidence produced by the experimental infections that a very virulent organism of the hemorrhagic septicemia group probably highly adapted to the rat tissues was responsible for the lesions found in the wild rats from certain localities in Oakland. The body strains possess greater invasive properties than the culture strains. Inoculations in series invariably enhanced the invasiveness through the mucous membranes but primarily for the animal through which the passages were made, thus infectious tissues of guinea-pig passages were virulent when placed on the nose of these rodents but killed rats only on subcutaneous injection. The reverse was the case for passage material from rats. These important observations deserve further intensive study.

Toxin Production.—Heated cultures or filtrates are highly toxic for guinea-pigs and rabbits.

For example, a 72 hours' old hormone blood agar slant culture was washed off with phenolized salt solution; the suspension was heated for one hour at 56 C. The dense suspension showed completely dissolved organisms. One cc. contained enough toxin to kill a guinea-pig (subcutaneous injection) and a rabbit (intravenous injection). The Berkefeld filtrate of an 18 days hormone broth culture (shallow layer) was exceedingly toxic; amounts of 0.1 cc. injected intracutaneously killed guinea-pigs in 24 to 36 hours. Aggressins sterilized at 56 C. are toxic when inoculated subcutaneously in 0.5 to 1.0 cc. amounts. The toxemia manifests itself in form of a general lethargy and moderate edema at the site of the inoculation. Formaldehyde destroys the toxin when the filtrates are kept at room temperature for 48 hours.

These observations are not in harmony with those reported by Stang, Citron and Pütz, Hadley [29] and others for the other members of the hemorragic septicemia group. The reasons for these discrepancies deserve further investigation in the light of the toxin studies on streptococci (Dick,[30] Birkhaug [31]) and pneumococci (Zinsser and Grinnell [22]).

Immunity.—Guinea-pigs repeatedly treated with 2 to 5 cc. of antiplague serum (Pasteur and Swiss Serum Institutes) succumbed to 0.1 cc. of a young broth culture of the hemorrhagic septicemia organism in the same time as the controls. Properly conducted prolonged immunization with aggressins, a strain of P. avisepticum highly virulent for pigeons but not for guinea-pigs and heated cultures, protect guinea-pigs and rats against any one of the 34 strains encountered in the course of this survey. However, the immune rodents remain susceptible for P. pestis introduced cutaneously or subcutaneously. This is an additional proof that the bacilli are not true plague organisms. In a separate paper the difficulties which are encountered in producing a permanent immunity in guinea-pigs will be detailed.

DISCUSSION

The morphologic and cultural evidence, and the results of the animal experiments place the micro-organism in the hemorrhagic septicemia group. Certain biochemical reactions, the intolerance to gentian violet and in particular the agglutination tests afford a rapid and trustworthy method for the differentiation of the organism from P. pestis. The anatomical lesions produced by the bacillus resemble those of plague and therefore confuse the routine diagnosis of this disease in rats. Wayson [1] has discussed certain aspects of the problem and the writers have amplified his report in the introduction to the first paper of this series. Laboratory procedures, cultivation on gentian violet agar and immuniza-

[29] J. Bact., 1918, 3, p. 278.
[30] J. Am. Med Sci., 1925, 84, p. 802.
[31] Bull. Johns Hopkins Hosp., 1925, 37, p. 85.
[32] Proc. Soc. Exper. Biol. & Med., 1925, 23, p. 205.

tion of guinea-pigs, which have been developed, greatly facilitate and expedite the differential diagnosis. In this connection attention should, however, be called to the fact that 24 of a series of 88 rats carefully studied at necropsy presented lesions suggestive of hemorrhagic septicemia, plague, or of both and neither animal inoculations nor cultures confirmed the macroscopic routine diagnosis. It is quite likely that some of the lesions attributed to the pasteurella organism were probably those of rat typhoid due to either B. aertrycke or B. enteritidis. But the large percentage of lesions which bacteriologically remained undiagnosed should furnish sufficient argument to those who wish to perfect the technic of the plague laboratory.

A perusal of the many extensive reports on the routine diagnosis of rodent plague conveys the impression that infections due to organisms of the pasteurella group other than plague may interpose great difficulties. However, a detailed description of the disease or of the bacteriological criteria could not be found. In fact, the Indian Plague Commission dismisses the subject with the statement that the differentiating test of the pasteurella group was furnished by the employment of MacConkey's medium on which the hemorrhagic septicemia organism (swine plague organism) is definitely inhibited. As already mentioned, Neumann [25] describes and illustrates the pathologic and bacteriologic findings which he made on one rat. This animal was the only one which died suddenly in a group of wild rats kept in the laboratory for diagnostic work. Smillie [26] on the other hand encountered pasteurella organisms from normal rats of São Paulo, at a time when the city was in close proximity to and in constant danger from plague. On account of the morphologic similarity of the bacilli found in the rats to P. pestis a painstaking differential study was made, and although the strains isolated at São Paulo are not identical with that studied by Neumann, both workers proved that the bacilli were not plague but belonged to the closely allied pasteurellosis group.

Indirect evidence that wild rats may harbor hemorrhagic septicemia organisms is evidenced by the publication of Kister and Schmidt [8] who studied a highly fatal septicemia among ferrets. These animals were used as rat catchers on the piers of the harbor of Hamburg. Suddenly approximately 30 of the ferrets succumbed to an infectious disease. Bacteriologic examinations revealed a plaguelike bacillus which was subsequently identified as a representative of the bipolaris group. In its morphologic and cultural characteristic the ferret bacillus is identical with the rat organism described in this paper. However, it was highly

pathogenic for rats on feeding, killed cats and small birds. Furthermore, the pathogenicity was rapidly lost irrespective of repeated animal passages. Kister and Schmidt in the light of their observations discuss the possibility of epidemics among rats due to pasteurella bacteria which anatomically and bacteriologically might suggest plague. Their premonition has come true as the data of this paper fully exemplify.

The organism is highly virulent for laboratory animals and may therefore be used to great advantage in immunologic and epidemiologic studies on white rats. When properly adapted it is readily transmitted by contact and it is reasonable to assume that the epidemic which occurred among the wild rats of Oakland, California and the neighboring cities spread in a manner similar to a respiratory disease. How it originated will remain unknown but as soon as an opportunity is afforded, a search for carriers will be instituted among the rodents of a locality in which the incidence was very high. In fact a number of important problems still await an investigation, in particular the sanitary significance deserves some consideration. Until recently the pathogenicity of the members of the hemorrhagic septicemia group was not appreciated. The literature contains reports on laboratory infections with fowl cholera cultures (Kolle and Hetsch,[33] Kitt [7] and others), but recently a number of cases due to pasteurella organisms have appeared in French and German publications (v. Boer,[34] Martin and Höpli,[35] Leger and Baury,[36] Kernéis,[37] Teissier, Gastinel, Reilly and Rivalier.[38] It is not the place to analyze the evidence on which the diagnoses were placed. The bacteriologic criteria which in some papers were chosen to classify the isolated micro-organisms in the pasteurella group are not compatible with our present knowledge. The report by Teissier, Gastinel, Reilly and Rivalier on a fatal case of pleurisy with positive pasteurella findings is the most convincing, although little information is given concerning the source of the infection.

Finally the classification of the organism deserves some consideration. As repeatedly indicated the evidence here presented places the bacillus into the hemorrhagic septicemia group. According to Bergey [39] it would be classified with the Genus XIX, Pasteurella. It is not the place to discuss the most unsatisfactory criteria which are used in this publi-

[33] Exper. Bakteriol. u. Infektionskrankh., 1915, p. 650.
[34] Centralbl. f. Bakteriol., I, O., 1917, 79, p. 390.
[35] Centralbl. f. Bakteriol., I, O., 1923, 90, p. 241.
[36] Bull. Soc. de path. exot., 1922, 15, p. 495.
[37] Ibid., 1922, 15, p. 491.
[38] J. physiol. et path. gen., 1922, 22, pp. 212, 241.
[39] Manual of Determinative Bacteriology, p. 266.

cation to identify the species, but attention is called to the error which was made in the compilation of this genus. As species 2, Pasteurella muriseptica is described as an organism which was originally studied and fully reported as Bacillus murisepticus by Flügge [40] in 1886. The description given by this worker, also partially copied and somewhat extended by Migula [41] and restudied by Lehmann and Neumann [42] under the name Bacterium murisepticum (Flügge) Migula, has very little in common with the representatives of Pasteurella. It retains the gram stain and forms arborescent colony aggregations in deep gelatin and resembles Erysipelothrix rhusiopathiae.

Many years ago one of the writers (K. F. M.) has investigated a septicemia of mice and has isolated an organism which fulfils the criteria given by Flügge and Migula. Even with the greatest imagination it is impossible to consider the bacterium of Flügge under Pasteurella; the species 2 in Bergey's Manual should therefore be transferred to where it belongs, probably the genus IV, Erysipelothrix, of the family Actinomycetaceae Buchanan.

In order to classify the organism here described, a new species, Pasteurella muricida (N. Sp.), with the following characteristics is proposed as an addition to the genus Pasteurella:

Rods: 0.3 to 0.75 to 0.2 micron, occurring singly or in short chains (3 to 4 individuals) nonmotile; gram-negative.
Gelatin: Small, delicate smooth edged, whitish colonies.
Gelatin Stab: Translucent filiform, granular growth without lateral projections in stab. Fine whitish surface growth, no liquefaction.
Agar Colonies: Circular dewdrop-like whitish colonies with even borders, becoming opaque in the center and more transparent toward the edges; often viscid and removable en bloc.
Agar Slant: Thin, grayish, stringy film.
Broth: Slight turbidity with stringy, slimy to flaky coarse sediment and fine pellicle formation.
Bromcresol Purple Milk: Unchanged or slight increase in H-ion concentration.
Potato: No growth.
Indol formed; nitrates reduced.
Acid in dextrose, levulose, galactose but not in saccharose or lactose.
Aerophilic.
Optimum temperature 30 to 37 C.
Habitat: Septicemia or focal lesions in wild rats resembling plague. Pathogenic for guinea-pigs, rabbits, mice, white and gray rats, but not for chickens and pigeons.

SUMMARY

A detailed pathologic, anatomic and bacteriologic study of 88 wild rats caught in the course of plague control work in Oakland and Alameda, California, proved the existence of four rodent diseases, hemorrhagic septicemia (pasteurella), plagus, rat typhoid (due to B. enteritidis and B. paratyphosus B) and pseudotuberculosis (due to B. pseudotubercu-

[40] Die Mikroorganismen, 1886, p. 250.
[41] System d. Bakterien, Jena, 1900, 2, p. 433.
[42] Atlas and Grundriss d. Bakteriologie 1912, Part 2, p. 428.

losis rodentium Pfeiffer). The comparative anatomic findings and the significance of the diagnostic guinea-pig inoculations and cultures are discussed.

The causative organism of the hemorrhagic septicemia for which the name, Pasteurella muricida (N. Sp.) is proposed, resembles morphologically, culturally, biochemically and serologically the well known representatives of the pasteurella group. It is highly pathogenic for guinea-pigs, rabbits, mice, and white and gray rats. Barnyard birds and cats are refractory. A spontaneous epidemic among laboratory rats introduced by an animal experimentally infected by the nasal route is reported.

SOME INFLUENCES OF ANTITOXIC AND OTHER SERUMS ON BOTULINUM INTOXICATION *

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It is well known that various strains of C. diphtheriae may differ more or less in agglutinability, fermentative powers, stability of suspensions, electrophoretic potential difference and toxigenicity, but the toxins produced by this species are the same antigenically and produce identical physiologic and anatomic effects. It is also generally maintained that toxins produced by single-cell cultures of Cl. botulinum are neutralized only by their homologous type of antitoxin. Heterologous botulinum antitoxins as well as such other antitoxins as those of tetanus or diphtheria are considered impotent in preventing experimental botulism. Apparently the only bacteria in which strains do not form antigenically identical toxins are Cl. botulinum and the hemolytic streptococci of scarlet fever. Park and Spiegel [1] observed that the scarlet fever toxic filtrate produced by a single strain was not a single toxin but a group of toxins. Kirkbride and Wheeler [2] reported that an individual had been found who gave a cutaneous reaction with the Dick or Williams toxin and whose serum neutralized the Dochez toxin. Falk and Powdermaker [3] working with Cl. botulinum produced agglutinating serums with acidified and basic antigens which showed heterologous as well as homologous agglutinating potencies for strains of the antitoxic A and B types. It appeared from their work that the antigenic differences between the A and B types were of a quantitative rather than qualitative nature. Schoenholtz and Meyer [4] have frequently noted marked cross agglutination between A and B types. However, no report has been made of other than qualitative differences of the botulinum toxins A and B.

The work reported here was undertaken to determine whether or not large quantities of nonspecific serums would influence the potency of a small dose of botulinum toxin.

Preparation of Materials.—Toxins were prepared in beef heart medium from single-cell cultures of Cl. botulinum. Sixteen strains were used—10 type A, and

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[1] J. Immunol., 1925, 10, p. 829.
[2] Proc. Soc. Exper. Biol. & Med., 1924, 22, p. 85.
[3] J. Infect. Dis., 1925, 37, p. 514.
[4] J. Immunol., 1925, 10, p. 1.

6 type B. The initial reaction was P_H 7.8. The vaseline sealed tubes were incubated for 10 days at 37 C. The toxic broth was freed from cells by being drawn through Mandler filters and also, in some instances, by centrifugation at high speed. Types A and B botulinum antitoxins were obtained from sheep which had been immunized with toxins produced by single-cell strains of Cl. botulinum (type B, strain 126.7; type A, strain $M7A_2$). Standard botulinum antitoxins were obtained from the U. S. Public Health Service, Hygienic Laboratory, Washington, D. C.

Because of certain superficial similarities between type B cultures of Cl. botulinum and Cl. sporogenes, a sporogenes filtrate antiserum was prepared by giving rabbits 10 intravenous injections of the filtrate of a 10 day beef heart culture with intervals of 5 days between each injection. Each animal received a total of 35 cc. of filtrate during the immunization.

Normal rabbit and sheep serums were obtained from the animals which were to be used for the production of antiserums. Whole diphtheria and tetanus antitoxic serums were obtained from commercial laboratories.

Experimental.—The technic, unless otherwise indicated in the specific protocol of an experiment, was as follows:

The MLD of toxin was determined for white mice, using for criteria of toxicity four dilutions of toxin for each order of magnitude, and death of the mouse in 48 hours. For guinea-pigs the usual criteria were used, i. e., smallest amount of toxin introduced subcutaneously which kills a 250 gram guinea-pig in 4 days. Immediately after this determination each toxin was used as indicated in the tables. Measured doses of toxin and various quantities of serum were drawn into syringes, mixed and allowed to stand at 20 to 22 C. for 1 hour before injection intraperitoneally into white mice, or subcutaneously into 250 gram guinea-pigs. In conjunction with the experiments with incubated toxin-serum mixtures, toxin was injected intraperitoneally into white mice one hour previous to the administration of serum and conversely serum was given to another series of mice one hour before toxin was injected. In each experiment all of the animals received the same volume of liquid. Sterile 0.85% NaCl solution was used as diluent.

TABLE 1

EFFECT ON GUINEA-PIGS OF SUBCUTANEOUS INJECTIONS OF TOXIN-ANTITOXIN MIXTURES THAT HAD BEEN IN CONTACT FOR 1 HOUR

	Number Guinea-Pigs (250-300 Gm.)	Toxin 1 cc. Type	Toxin 1 cc. Dilution	MLD	Antitoxin Units (0.4 cc. = 1 unit)	Type	Results
Exper. 1.	7	A ($M7A_2$)	1:20,000	1	0.1	B	Guinea-pigs died in 5 to 8 days (1 in 14 days)
				10	0.1		
				1	0.2		
				1	0.3		
				1	0.4		
				1	1.0		
				1	0.0		
	7	A (KIA18)	1:4000	(Same mixtures as above)		B	Death in 2 to 3 days
	7	A (DIA)	1:1000	(Same mixtures as above)		B	Death in 4 to 7 days, 1 within 12 hours
Exper. 2.	7	B (B64)	1:3000	(Same mixtures as above)		A	5 lived, 2 (10 MLD and no antitoxin) died in 5 and 6 days
	7	B (126.7)	1:1500	(Same mixtures as above)		A	5 lived, 2 (10 MLD and no antitoxin) died in 2 and 4 days

Total amount injected, 4 cc.; dilutions in 0.85% NaCl solution.

From experiment 1 it appears that type B antitoxin has no power to nullify the action of 1 MLD of type A toxin when introduced subcutaneously into a guinea-pig. In contrast are the results in experiment 2 showing nullification of type B toxin when mixed with many "equivalents" of type A antitoxins. The normal serums from these sheep were without antitoxic properties for the various toxins of Cl. botulinum as will be shown later.

TABLE 2

EFFECTS ON WHITE MICE OF INTRAPERITONEAL INJECTIONS OF TOXIN-ANTITOXIN MIXTURES INCUBATED 1 HOUR AT 20 C.

	Number of Mice	Toxin, 0.5 cc. Type	Toxin, 0.5 cc. Dilution	MLD and Antitoxin Units		Antitoxin Type, Units per Cc.	Results Symptoms (Hours)	Results Death (Hours)
Exper. 3.	5	A (1592.2)	1:1250	1	0.1	B, 140	..	All (25) died within 48 hours
				10	0.1			
				1	0.5			
				1	1.0			
				1	0.0			
	5	A (84A1)	1:1250	(Same as above)		B, 140		
	5	A (SA12)	1:250	(Same as above)		B, 140		
	5	A (A27)	1:500	(Same as above)		B, 140		
	5	A (B7af)	1:4000	(Same as above)		B, 140		
	(Total injection, 1 cc.)							
Exper. 4.	5	A (1592.2)	1:1250	1	0.1	C, 20	..	24
				10	0.1	.....	..	24
				1	0.5	.....	..	24
				1	1.0	.....	..	24
				1	0.0	.....	24	48
	5	A (84A1)	1:1250	(Same as above)		C, 20	24	48
							..	24
							..	48
							24	48
							..	48
	5	A (M6A)	1:500	(Same as above)		C, 20	..	24
							..	24
							..	24
							..	24
							24	48
	5	A (SA12)	1:250	(Same as above)		C, 20	24	48
							24	48
							24	48
							24	48
							..	48
	5	A (M6D)	1:375	(Same as above)		C, 20	..	24
							..	24
							..	24
							24	48
							24	48
Exper. 5.	4	B (276B1)	1:100	1	0.1	C, 20	24	72
				1	0.5		..	Survived
				1	1.0		..	48
				1	0.0		24	48
	4	B (B_2HB27)	1:100	(Same as above)		C, 20	..	Survived
							..	Survived
							..	Survived
							24	48
	4	B ($A412_2$S)	1:250	(Same as above)		C, 20	..	Survived
							..	Survived
							..	Survived
							24	48
	4	B (1500B)	1:500	1	0.1	C, 20	..	Survived
				1	0.5		..	Survived*
				1	1.0		..	48

TABLE 2—Continued

EFFECTS ON WHITE MICE OF INTRAPERITONEAL INJECTIONS OF TOXIN-ANTITOXIN MIXTURES INCUBATED 1 HOUR AT 20 C.

	Number of Mice	Toxin, 05 cc. Type	Toxin, 05 cc. Dilution	MLD and Antitoxin Units		Antitoxin Type, Units per Cc.	Results Symptoms (Hours)	Results Death (Hours)
Exper. 6.	4	B (B_2HB27)	1:100	1	0.1	A, 200	48	72
				1	0.5		24	Survived
				1	1.0		..	Survived
				1	0.0		24	60
	4	B (A412.5)	1:250	(Same as above)		A, 200	24	30
							24	Survived
							..	Survived
							24	48
	4	B (1500B)	1:500	(Same as above)		A, 200	24	30
							72	5 days
							..	Survived
							..	24
	4	B (276B1)	1:100	(Same as above)		A, 200	24	48
							..	Survived
							..	Survived
							..	48
	4	B (B64)	.....	(Same as above)		A, 200	24	72
							24	48
							48	96
							..	48
	7	B (126.72)*	1:1000	1	0.1	A, 200	..	24
				1	0.5		..	Survived
				1	1.0		..	Survived
				1	2.0		..	Survived
				2	2.0		..	24
				1	5.0		..	Survived
				1	0.0		..	24
	6	B (126.71)	1:100	1	0.1	A, 200	48	72
				1	0.5		..	Survived
				1	1.0		..	Survived
				1	2.0		..	Survived
				2	5.0		..	72
				1	0.0		..	24

* Toxin freed from bacteria by centrifugation.

It is evident from experiment 3 that type B antitoxin exerts no protective influence against 1 MLD of type A toxin. In a few instances adjuvant reactions occur. This is especially marked with toxin B7af. One MLD of four type A toxins not listed (M6A, M6D, A5f and A320) when mixed with B antitoxin affected white mice in the same manner as is shown for toxin SA12.

Experiment 4 shows that type C antitoxin enhances the toxicity of 1 MLD of toxin A in many instances. These adjuvant properties of type C antitoxin were also observed with four other type A toxins not listed in these tables.

Experiment 5 shows that type C antitoxin nullifies the toxicity of 1 MLD of type B toxin in a few instances. However, with three B toxins not listed nullification of toxicity did not occur when type C antitoxin was given as indicated in this experiment.

It appears from experiment 6 that incubated mixtures of B toxin-A antitoxin are often innocuous. However, it will be noted that the action of toxin B64 was not nullified although the potency was reduced somewhat by large quantities of type A antitoxin. Subsequent experiments with fresh toxin formed by strain B64 showed that nullification took place with type A antitoxin.

TABLE 3

TOXINS AND ANTITOXINS INJECTED INTO MICE AT DIFFERENT TIMES

	Number of Mice	Toxin, 1 cc.		MLD and Antitoxin A, Units		Results	
		Type	Dilution			Symptoms (Hours)	Death (Hours)
Exper. 7..... Type B toxins injected intraperitoneally 1 hour before type A antitoxin	6	B (126.71)	1:200	1	0.1	24	48
				1	0.25	..	Survived
				1	0.5	..	48
				1	1.0	..	Survived
				2	1.0	..	24
				1	0.0	..	24
	5	B (A41225)	.....	1	0.1	24	48
				1	0.5	24	48
				1	1.0	48	72
				1	2.0	..	Survived
				1	0.0	24	48
	5	B (BHB27)	.....	(As above)		24	48
						48	72
						..	Survived
						..	6 days
						24	48
	5	B (1500B)	.....	(As above)		24	48
						24	48
						48	72
						24	72
						24	48
	5	B (276B1)	1:100	(As above)		24	48
						24	48
						48	72
						24	72
						24	48
	5	B (B64)	1:250	(As above)		24	48
						None	Survived
						48	6 days
						..	48
						24	48
	6	B (126.7)	1:100	1	0.1	24	48
				1	0.5	36	48
				1	1.0	None	Survived
				1	2.0	24	48
				2	2.0	..	24
				1	0.0	24	48
	7	B (126.72)*	1:1000	1	0.1	..	48
				1	0.5	..	48
				1	1.0	..	24
				1	2.0	..	48
				2	2.0	..	24
				2	2.0	..	24
				1	0.0	24	48
	6	B (126.7R)*	1:1000	1	0.1	..	24
				1	0.5	..	24
				1	1.0	..	48
				1	2.0	..	24
				2	2.0	..	24
				1	0.0	..	24

* Control mice for 1 MLD in experiment 7.

TABLE 3—Continued

TOXINS AND ANTITOXINS INJECTED INTO MICE AT DIFFERENT TIMES

	Number of Mice	Toxin, 1 cc. Type	Toxin, 1 cc. Dilution	MLD and Antitoxin	A, Units	Results Symptoms (Hours)	Results Death (Hours)
Exper. 8..... Type B toxin administered 1 hour after type A antitoxin	5	B (126.71)	1:200	1	0.1	..	24
				1	0.5	..	24
				1	1.0	24	Survived
				1	2.0	24	48
				2	2.0	24	48
	4	B ($A_4 12_2 5$)	1:25	1	0.1	24	48
				1	0.5	24	48
				1	1.0	24	48
				1	2.0	24	48
	4	B (BHB27)	1:50	(As above)		..	24
						24	48
						..	Survived
						72	5 days
	4	B (B64)	.....	(Same as above)	A, 200	25	72
						24	48
						24	48
						24	48
	4	B (276B1)	1:100	(As above)		..	24
						..	48
						24	Survived
						48	4 days
	4	B (B64)	1:250	(As above)		48	72
						48	Survived
						..	Survived
						24	48
	5	B (126.7)	1:100	1	0.1	48	96
				1	0.5	72	120
				1	1.0	..	Survived
				1	2.0	48	Survived
				2	2.0	24	30
	6	B (126.72)*	1:1000	1	0.1	..	24
				1	0.5	..	24
				1	1.0	..	24
				1	2.0	24	48
				2	2.0	24	48
				2	2.0	24	Survived
	5	B (126.72)*	1:1000	1	0.1	24	48
				1	0.5	..	24
				1	1.0	..	24
				1	2.0	..	24
				2	2.0	..	24

* Freed from bacteria by centrifugation at high speed.

It is evident from experiments 7 and 8 that the injection of 1 MLD of type B toxin into mice and subsequently administering type A antitoxin, or the converse, results in death of the animal in most instances, unless large quantities of antitoxin are given. Fewer equivalents of the heterologous antitoxin are needed to nullify 1 MLD of type B toxin when these B toxin-A antitoxin mixtures are incubated before injection intraperitoneally, as will be observed in experiment 6.

TABLE 4

EXPER. 9.—NORMAL SHEEP SERUM AND BOTULINUM TOXINS INCUBATED TOGETHER AT 22 C. FOR 1 HOUR BEFORE INTRAPERITONEAL INJECTION INTO WHITE MICE

Number of Mice	Toxin 0.5 Cc. Type B (126.7) Dilution	MLD	Normal Sheep Serum		Results	
			Cc.	Dilution	Symptoms (Hours)	Death (Hours)
7	1:100	1	0.1	1:100	24	48
	1:100	1	0.25	1:50	24	48
	1:100	1	0.5	1:50	24	72
	1:100	1	1.0	1:50	24	48
	1:100	1	1.0	1:25	..	24
	1:50	2	1.0	1:10	..	24
	1:100	1	0.0	.....	24	48
	Type A (M7A2)					
7	1:7500	1	0.1	1:140	..	48
	1:7500	1	0.25	1:70	..	48
	1:7500	1	0.5	1:70	48	72
	1:7500	1	1.0	1:70	48	72
	1:7500	1	1.0	1:70	..	48
	1:7500	2	1.0	1:10	..	24
	1:7500	1	0.0	.....	24	48

Total amount injected into mouse, 2 cc.

In conjunction with experiment 9 normal sheep serum in the dilutions shown was given intraperitoneally 1 hour before types A and B toxins. An enhancement of toxicity was especially marked in 10 mice where high concentrations of serum were used with both types of toxin. When toxins were injected 1 hour before normal sheep serum this reinforcing action was again observed in most of the animals (6 in 10) receiving more concentrated serum.

TABLE 5

EXPER. 10.—BOTULINUM TOXIN AND TETANUS ANTITOXIN INCUBATED TOGETHER 1 HOUR AT 22 C. BEFORE BEING INJECTED INTRAPERITONEALLY

Number of Mice	Toxin 1 Cc. Type B (126.7) Dilution	MLD	Tetanus Antitoxin		Results	
			Cc.	Dilution	Symptoms (Hours)	Death (Hours)
7	1:1000	1	0.1	1:100	..	48
	1:1000	1	0.25	1:50	..	48
	1:1000	1	0.5	1:50	..	24
	1:1000	1	1.0	1:50	..	24
	1:500	2	1.0	1:25	24	36
	1:1000	1	1.0	1:10	36	48
	1:1000	1	0.0	.....	..	48

In conjunction with experiment 10, 5 mice were given 1 MLD of toxin an hour before the nonspecific antitoxin and 5 received the serum 1 hour before toxin. Adjuvant action was manifested in both series when high concentrations of serum were used.

TABLE 6

EXPER. 11.—DIPHTHERIA ANTITOXIN AND BOTULINUM TOXIN TYPE B INCUBATED TOGETHER AT 22 C. FOR 1 HOUR BEFORE INTRAPERITONEAL INJECTION

Number of Mice	Toxin 1 Cc. Type B (126.7) Dilution	MLD	Diphtheria Serum		Results	
			Cc.	Dilution	Symptoms (Hours)	Death (Hours)
8	1:2000	1	0.1	1:100	24	48
	1:2000	1	0.25	1:50	24	48
	1:2000	1	0.5	1:50	24	62
	1:1000	2	1.0	1:50	..	24
	1:2000	1	1.0	1:25	24	62
	1:2000	1	1.0	1:10	..	24
	1:2000	1	1.0	Undiluted	..	24
	1:2000	1	0.0	.....	24	48

Whole diphtheria antitoxic serum manifests itself as an adjuvant substance. High concentrations of the serum only were effective in producing this reaction.

TABLE 7

EXPER. 12.—BOTULINUM TOXIN AND NORMAL RABBIT SERUM INCUBATED TOGETHER 1 HOUR AT 22 C. BEFORE INTRAPERITONEAL INJECTION

Number of Mice	Toxin 1 Cc. Type B (126.7) Dilution	MLD	Normal Rabbit Serum		Results	
			Cc.	Dilution	Symptoms (Hours)	Death (Hours)
7	1:2000	1	0.5	1:50	24	48
		1	1.0	1:25	..	48
		1	0.5	1:10	24	48
		1	1.0	1:10	..	24
		1	0.25	Undiluted	..	48
		1	1.0	Undiluted	..	24
		1	0.0	.....	24	72

This experiment shows that normal rabbit serum has no nullifying power over 1 MLD of type B toxin. The intensifying action of the serum is apparent.

TABLE 8

EXPER. 13.—INTRAPERITONEAL INJECTION OF SPOROGENES FILTRATE ANTISERUM AND TYPE B TOXIN INCUBATED TOGETHER 1 HOUR AT 22 C.

Number of Mice	Toxin 1 Cc. Type B (126.7) Dilution	MLD	Sporogenes Filtrate Rabbit Antiserum		Results	
			Cc.	Dilution	Symptoms (Hours)	Death (Hours)
12	(11) 1:2000	1	0.5	1:50	..	48
		1	1.0	1:50	..	48
		1	1.0	1:25	..	24
		1	0.5	1:25	..	48
		1	1.0	1:10	..	24
		1	0.5	1:10	..	24
		1	0.1	Undiluted	..	24
		1	0.25	Undiluted	..	24
		1	0.5	Undiluted	..	24
		1	1.0	Undiluted	..	24
		1	0.0	Control	24	48
	0 Control	0	1.0	Undiluted	None	Survived

This experiment presented the most striking instances of adjuvant action.

DISCUSSION

In no instance did a type B botulinum antitoxin influence the lethality of 1 MLD of type A toxin in any way except in enhancing intoxication when white mice were used as test animals. When guinea-pigs were used this adjuvant action of the heterologous type B serum was not especially marked with 1 MLD of type A toxin. That this mixture is more poisonous than the toxin alone is the belief of Meyer, Wagner and Dozier.[5] Bitter [6] also cites instances in which botulinum toxin is rendered more active for guinea-pigs by heterologous sera, nonspecific serums, normal horse serum and soap solution. The intensifying action of B and C antitoxins in type A intoxication should be taken into consideration in evaluating the status of serum therapy of botulism.

Incubated mixtures of type A toxin + type C antitoxin kill mice regularly; with high concentrations of serum marked intoxication occurs with but 1 MLD of toxin. However, with type B toxin a definite lessening of toxicity occurs in a few instances when 1 MLD is used as a test dose. Adjuvant action in mice was not observed with B toxin + C antitoxin mixtures.

The toxicity of 1 MLD of type B toxin mixed with 0.5 to 2.0 units of type A antitoxin was usually nullified either in incubated mixtures or when this toxin was given one hour before or after administering the heterologous antitoxin. 100 units of type A antitoxin failed to protect guinea-pigs from 2 to 5 MLD of type B toxin. Adjuvant action was not observed in guinea-pigs or white mice with these mixtures of toxin B and antitoxin A.

With normal rabbit and sheep serums nullification of toxicity did not occur in a single instance. The adjuvant action of these serums was always apparent. Tetanus and diphtheria whole serum acted much the same on the course of intoxication as did normal serums. The most striking adjuvant action occurred with sporogenes filtrate antiserum and type B toxin.

Although these toxins are considered to be alike only through their ability to produce identical physiologic and anatomic effects it appears from these experiments that a relationship of a sort exists between type A antitoxin and B toxin and between C antitoxin and B toxin inasmuch as nonspecific and normal serums increase intoxication rather than nullify or lessen the toxicity of the MLD of toxin.

[5] J. Bact., 1925, 10, p. 321.
[6] Ergebn. d. allg. Path. u. path. Anat., 1921, 19, (2), p. 733.

The adjuvant action of protein substances was first studied by Ricketts and Kirk.[7] Using salted out tetanus toxin they found that several substances—whole normal goat serum, rabbit serum, egg albumin and beef broth—all intensified the intoxication in white mice. It is a noteworthy fact that a salted out toxin becomes more potent when dissolved in beef infusion broth before being injected into test animals. In terms of the Ehrlich nomenclature, they explained the phenomenon of adjuvant action to be due to a nonspecific engagement of the activities of the cells without a direct occupation of toxinophile receptors. Besredka's [8] experiment with leukocytes is an example of such a process. He observed that when the cells were filled with carmine granules they did not have the ability to take up a subsequent injection of arsenic with the result that animals died more readily from arsenic poisoning. Von Dungern [9] also noted this interference of adsorption of a second substance. By intravenous injections of egg albumin or of plasma of octopus vulgaris into a rabbit the ability to absorb a subsequent injection of the plasma of the spider crab was generally inhibited.

The experiments of Julian Lewis [10] show that when small quantities of one protein are injected into a guinea-pig together with large quantities of another protein the small quantities fail to sensitize or manifest themselves as antigens. Hence in case of injecting a toxin and too little antitoxin or a whole serum poor in antitoxin the defensive mechanism of the body might not show maximum resistance to the poison.

It is barely possible that a few cases of serum sickness in toxemia in man may be due to adjuvant action. Zinsser [11] made the following comments: "Joint pains commonly accompany the appearance of the rash, etc. . . . They are not often severe in actual discomfort; but may lead to a stiffness and difficulty in motion in cases in which tetanus antitoxin has been administered, giving rise to a considerable amount of worry. The writer has seen two cases in which the joints of the jaws, of the back of the neck, and of the legs were so affected that slight rigidity and in one instance the difficulty of easily opening the mouth caused a good deal of uncertainty as to whether serum sickness or early tetanus were involved. . . . If serum sickness is truly an anaphylactic phenomenon, however, it is still by no means clear why symptoms should at all ensue after the first injection."

[7] J. Infect. Dis., 1906, 3, p. 115.
[8] Ann. de l'Inst. Pasteur, 1899, 12, p. 49.
[9] Die Antikörper, 1903. Cited from Ricketts and Kirk.[7]
[10] J. Infect. Dis., 1915, 17, p. 241.
[11] Infection and Resistance, 1923, p. 470.

SUMMARY

The toxicity of 1 MLD of type B botulinum toxin is commonly nullified when mixed and incubated at 20 C. for 1 hour with large amounts of type A antitoxin before introduction into test animals or when the toxin and serum are given separately within an interval of 60 minutes.

Large quantities of type C antitoxin lessen the potency of 1 MLD of type B toxin.

Type A toxin seems to be neutralized only by its homologous antitoxin.

An adjuvant action is elicited in white mice by the following mixtures: type A toxin + B antitoxin, type A toxin + C antitoxin, and type A and B toxins with diphtheria or tetanus whole antitoxins, sporogenes filtrate antiserum, and normal rabbit or sheep serums.

INCIDENCE AND THERAPEUTIC VALUE OF STAPHYLOCOCCUS BACTERIOPHAGE IN ANTRUM INFECTIONS

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Although the great majority of experiments reported on bacteriophage activity are concerned with the theoretic aspect of the question, numerous attempts have been made to determine the therapeutic value of the lytic agent. For the most part, the claims of d'Herelle [1] as to the efficacy of bacteriophage in the treatment of disease have not been confirmed by Davison,[2] Munter and Boenheim,[3] Topley [4] and others. The recent work of Frisch,[5] however, suggests that in localized infections, such as cystitis, bacteriophage may prove of value.

It was, therefore, thought of interest to determine the incidence of bacteriophage in sinus infections and to try the effect of local instillations of the lytic agent. The organism most frequently isolated from sinus infections is the staphylococcus. The occurrence of a lytic agent in pus active against staphylococcus has been known since the fundamental experiments of Twort [6] with micrococci isolated from vaccine pulp; d'Herelle in 1921 reported the isolation of a staphylococcus albus bacteriophage from the pus of an infected finger; Gratia [7] repeated Twort's work, and Callow [8] demonstrated bacteriophage action in pus obtained from boils. It thus seemed probable, in view of the large amount of pus present in chronic sinus infections from which staphylococci are recovered that a lytic agent might be present.

A series of 40 cases of such sinus infection was examined for the presence of bacteriophage. Acute, subacute and chronic infections were included in the group. All cases were examined at least twice and the majority of them three times. The pus obtained from the sinus washings was inoculated into broth, approximately 2 cc. of mucopurulent material into 8 cc. of meat extract broth (P_H 8). The mixture was incubated at 37 C for 6 to 12 hours, being thoroughly shaken from time to time. The pus in most instances was of such a character that it

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[1] The Bacteriophage. American translation, 1922.
[2] Am. J. Dis. Child., 1922, 23, p. 531.
[3] Ztschr. f. Kinderheilk., 1925, 39, p. 388.
[4] J. Hyg., 1925, 24, p. 17.
[5] Wien. klin. Wchnschr., 1925, 38, p. 839.
[6] Lancet, 1915, 2, p. 1241.
[7] Compt. rend Soc. de biol., 1921, 85, p. 25.
[8] J. Infect. Dis., 1922, 30, p. 643.

settled to the bottom of the tube and the supernatant fluid could be decanted and sterilized by filtration through a Berkefeld candle without preliminary centrifugation. The homologous strain of the staphylococcus was isolated from the same specimen of pus. Each filtrate was tested against a young broth culture of the homologous strain in the following manner:

	Sterile Broth	Staphylococcus Culture	Filtrate	Salt Solution
Tube 1.....	2 cc.	1 drop	0.5 cc.	.....
Tube 2 (control).....	2 cc.	1 drop	.....	0.5 cc.

The tubes were incubated at 37 C for 2 hours and then streaked on agar plates. After standing overnight at room temperature, the turbidity of tube 1 was compared with the control tube, and both tubes again plated. In most instances no inhibition of growth in tube 1 was observed at this time. Tube 1 was then centrifuged until clear and the supernatant fluid was tested in the same way as the original filtrate (tubes 1 and 2).

Plates were again streaked after 2 hours' incubation and again after standing 18 hours at room temperature.

In this way each filtrate was carried through a series of 5 tests. In each series the growth in the tube to which the supernatant of the foregoing series had been added, was compared with the growth in the control tube without filtrate. The plates in all instances were so streaked that discrete colonies were obtained. The individual colonies were examined under the microscope with a 32 mm. lens for irregularities in outline or pigmentation, indicating the action of a lytic agent. To test the lysogenic property of the colonies which appeared abnormal, they were restreaked on agar plates to see if similar colonies developed since a certain amount of irregularity in the outline of colonies may be produced by crowding or by slight roughness of the agar.

By the method outlined we examined 40 cases of antral infection from which staphylococcus was recovered. In 4 instances a bacteriophage active against the homologous strain was isolated. A brief summary of the course of the infection in these 4 cases will be given:

1.—Pansinuitis over a period of 6 years. The antrum was washed (10/5/25), and a considerable amount of muco-pus was recovered. A pure culture of staphylococcus aureus was obtained. Lytic colonies were observed after 2 serial transfers and partial inhibition of growth after the 4th transfer. The antrum was washed (10/14), a moderate amount of muco-pus recovered, no bacteriophage present (11/10). The antrum was clear (11/10). Muco-pus obtained from the antrum (12/5).

2.—Acute exacerbations occurred over a period of two years apparently due to a variety of organisms. The antrum was washed (10/8), a considerable amount of muco-pus found, and a mixed culture of pneumococcus type 4 and staphylococcus aureus isolated. Direct plating showed numerous lysogenic colonies of staphylococcus aureus. Complete inhibition of growth was observed after the 10th serial transfer. The antrum was washed (11/23) and lytic colonies were again observed on direct plating and complete inhibition of growth after the 6th transfer. The antrum washings (11/28) showed no evidence of lytic activity on this date nor during the month following.

3.—Sinus infections were present intermittently over a period of 10 years and continuous during the past 3 years. At the onset of each attack a pneumococcus type 4 was isolated in

pure culture. Subsequently, a mixed culture of type 4 pneumococcus and a hemolytic staphylococcus aureus were present. Toward the end of the attack, however, the staphylococcus alone was recovered. The antrum was washed (3/11) and a mixed culture of pneumococcus type 4 and hemolytic staphylococcus aureus was obtained. Lysogenic colonies were isolated directly from the staphylococcus culture at this time. The antrum washings (3/15) showed lysogenic colonies after 2 transfers and partial inhibition of growth after 8 transfers. The antrum washing (3/17) yielded a very small amount of muco-pus, no bacteriophage was recovered. On 3/22 the antrum was clear. On 3/25 muco-pus was again obtained from the right maxillary and frontal sinuses.

4.—Sinus infections intermittently present over a period of 4 years. The antrum washings (3/9) yielded a moderate amount of yellow pus, a hemolytic staphylococcus aureus was isolated. Lysogenic colonies were observed after the 1st transfer and partial inhibition after the 5th transfer. The antrum (3/11) was practically clear and on 3/16 entirely clear.

TABLE 1

TREATMENT OF 11 ANTRUM INFECTIONS WITH STAPHYLOCOCCUS BACTERIOPHAGE

	Bacteriophage Instillations					
Case	Number	Dates (1925)	Antrum	Cc.	Concentration	Remarks
5	1	11/14	Left	1	Undiluted	Antrum washed 9 weeks later: pus present, bacteriophage present, and no improvement
6	1	11/18	Right	1	Undiluted	Antrum washed 12 weeks later: no improvement
7	4	12/ 1 12/10 12/17 3/ 9/26	Left	1 (each time)	Undiluted	Antrum washed every week for 3 months: no improvement
8	5	11/23 12/ 1 12/10 12/17 12/26	Right	1 (each time)	Undiluted	Antrum washed every week for 3 months: no improvement
9	3	11/28 12/ 4 12/11	Both	2 (each time)	Undiluted	Antrum washed 12 times in 3 months: considerable pus at last washing; bacteriophage recovered; no improvement
10	4	11/17 11/25 11/28 12/ 5	Both Both Right Right	2 2 1 1	1:100 Undiluted Undiluted Undiluted	No improvement
11	5	11/19 11/25 12/11 12/18 12/26	Left Left	1 1 (each time)	1:100 Undiluted	No reduction in amount of pus; no improvement
12	5	11/13 11/20 11/27 12/ 4 12/11	Both Both Both Left Left	2 2 2 1 1	1:100 Undiluted Undiluted Undiluted Undiluted	No reduction in amount of pus: no improvement
13	1	12/11	Left	1	Undiluted	Antrum washed after 2 weeks: bacteriophage recovered; antrum cleared after 8 weeks
14	3	11/30 12/ 7 12/10	Both	2 (each time)	Undiluted	No improvement
15	1	11/21	Both	2	Undiluted	No improvement; no more instillations because of toxic effect of bacteriophage

The occurrence of B. coli bacteriophage in stool filtrate was first demonstrated by d'Herelle and subsequently has been corroborated by numerous workers. To our knowledge the isolation of a lytic agent in pus active against B. coli has not been reported. We, therefore, thought

it would be of interest to examine the pus obtained from B. coli sinus infections for the presence of bacteriophage. The results, however, in these 5 cases were entirely negative. Similarly d'Herelle has described a bacteriophage active against B. proteus in stool filtrates. The filtrates from B. proteus antral infections failed to give any evidence of lytic activity against B. proteus.

In 4 of 40 cases (10%) therefore, a bacteriophage active against the homologous strain was isolated. It is impossible to state whether in the cases in which no bacteriophage was isolated, lytic activity might have been demonstrated if a large number of heterologous strains had been used. In 11 of these negative cases tests against 2 or 3 heterologous strains, were uniformly negative. The bacteriophages isolated were active not only against the homologous strain but also against a large number of heterologous strains. Bacteriophage 1 was found to be extraordinarily active. Titrated by the method of Appelmans [9] after the 12th serial transfer complete inhibition occurred in a dilution of 1 : 10 billion. This bacteriophage was almost as active against 11 heterologous staphylococcus strains isolated from cases of long standing and was therefore chosen for therapeutic use. Bacteriophage 2, after the 10th transfer was found to have a titer of 1 : 100 only; after the 20th transfer the titer was found to be 1 : 1,000,000 against the homologous strain. The titer with heterologous strains, however, was definitely lower. The titer of bacteriophage 3 was 1 : 1000, and of bacteriophage 4 was 1 : 10,000.

Eleven cases of staphylococcus antral infections of long standing were selected for treatment with bacteriophage 1. Previous examinations for the presence of a lytic agent were all negative. Large quantities of bacteriophage were prepared:

100 cc. of beef extract broth PH was inoculated with 1 cc. of a 4 hour broth culture of staphylococcus 1 and 1 cc. of the lytic agent added. After two hours' incubation at 37 C. the flask was placed at room temperature. It was usually clear after 18 hours and was then filtered through a Berkefeld candle.

In each case the homologous strain of staphylococcus was isolated and its susceptibility to bacteriophage 1 tested. After washing out the antrum with sterile salt solution, 1 cc. of a 1 : 100 dilution of bacteriophage 1 was instilled. After one week the antrum was again washed with salt solution. At this time there was in most instances no reduction in the amount of pus present and the bacteriophage was recovered from the pus filtrate. Since no toxic effects were observed following the

[9] Compt. rend. Soc. de biol., 1921, 85, p. 1098.

instillation of the diluted bacteriophage in the first three cases it was considered safe to use the bacteriophage undiluted in subsequent cases. In one of the cases (15), asthmatic symptoms increased the day after the first instillation and no further treatments were given. It is possible that the exacerbation of asthma was due to the dissolved bacterial protein in the lytic solution. From one to five instillations of 1 cc. of the lytic agent were given at intervals of approximately a week. The amount and character of the antrum washings during the course of the treatment were carefully noted.

No improvement attributable to the instillation of the bacteriophage has been demonstrated in any of the 11 patients. In case 13, in which only one treatment was given, the sinus gradually cleared up in the course of 2 months and has remained clear for 8 weeks. It is extremely doubtful, however, whether the improvement in this case was due to the action of the lytic agent, since the patient has previously had similar periods of relief. In three of the cases (5, 6 and 9), in spite of the fact that the bacteriophage remained active over a considerable length of time, there was no reduction in the amount of pus. It was thought that possibly the antrums had failed to clear because the staphylococcus had become resistant to the lytic agent. Cultures of staphylococcus in 5 instances in which it was shown that bacteriophage had persisted for some time were isolated and their susceptibility to the bacteriophage tested. None of the direct streaks of these cultures on agar plates showed any lysogenic colonies. The titrations of these cultures with the lytic agent showed that none of these strains had become resistant.

CONCLUSIONS

In a series of 40 cases of antral infections from which staphylococci were cultured the presence of bacteriophage was demonstrated in 4 cases (10%). In 11 cases, the patients were given instillations in the sinuses with a very active lytic agent, but no improvement resulted.

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INVOLUTION FORMS OF ESCHERICHIA COLI

Arthur T. Henrici

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According to a theory recently proposed [1] to explain morphological variations in bacteria, the cells of bacteria undergo during the growth of a culture, a series of metamorphoses exactly analogous to those of a multicellular organism during the growth of an individual, and three distinct types may be recognized; an embryonic form, a mature form, and a senescent form. According to this view the so-called involution forms which appear in old cultures are analogous to the senescent forms in the cytomorphosis of a multicellular organism, and are just what their name implies, cells which have been acted upon by the lethal factor, whatever it may be, which limits growth, and which are dead or about to die and disintegrate. This has long been the view of the majority of bacteriologists, but has been vigorously opposed in recent years by several authors who have maintained that these unusual forms of bacteria are special reproductive cells of one type or another, phases in a rather complex life cycle.

The observations cited to support the above mentioned theory have been made for the most part during the early stages of growth, relatively little work having been done on the death phase. It has been shown that, whereas during the active growth period the cells show increased variability in size,[2] variability in form is a characteristic of the death phase,[3] but it is not possible from the work done so far to definitely correlate the degree of variation in form with the rate of death. The work here presented has had such an end in view.

The organism used in these studies is a typical strain of Escherichia coli obtained from the laboratories of the Minnesota State Board of Health in 1916 and which has been continuously cultivated on beef extract agar since. Five sets of 1.5% agar were used, the nutrient in each being 2% peptone. One set (P_H 7.4) had nothing further added. Another was made acid (P_H 4.5) by the addition of HCl, a third alkaline (P_H 9.2) by the addition of NaOH, a fourth had 5% NaCl added, and a fifth 3% $CaCl_2$ These mediums were chosen because they had been described by previous authors as giving rise to unusual cell types. They were distributed into measured test tubes of uniform diameter, each tube receiving 5 cc.

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[1] Henrici, A. T.: Science, 1925, 41, p. 644.
[2] J. Infect. Dis., 1926, 38, p. 54.
[3] Ibid., 1925, 37, p. 75.

of medium, and were slanted as nearly as possible at the same angle. Each tube was inoculated with one loopful of a 24 hour agar culture suspended in sterile water, spread as uniformly as possible over the surface. The cultures were incubated at 37 C. in a moist chamber to prevent drying of the medium. At the end of 1, 2, 3, 5, 7, 10, 14, 19 and 25 days samples were removed (the final samples of the NaCl series were removed on the 20th day, listed in table 2 as the 19th day). Five tubes of each medium were removed for sampling, the growth of each suspended in 5 cc. of sterile water, and the contents of the 5 tubes was then pooled. From this suspension a 1 cc. sample was removed and serially diluted in sterile tap water for plate counts. Plates were made in triplicate, the counts recorded being the mean of 3 plates of the same dilution, that dilution being chosen which gave the nearest to 100 colonies per plate. Another 1 cc. sample was mixed with an equal volume of 2% aqueous Congo red and a series of slides prepared for microscopic counting, using a technic essentially the same as that which I have previously described[4] save that the film was spread over 8 sq. cm. instead of 4, the thinner films giving more uniform results when large numbers of organisms are present. From these slides the proportion of dead cells stained by Congo red was also determined. Photomicrographs were made from these same slides, a number of different fields being photographed from each culture. The only criterion used in choosing the fields to be photographed was the uniformity of the distribution of the cells; otherwise they were chosen quite at random. The photographic negatives were projected onto paper, the final magnification being 19,000 diameters, and 200 cells were traced from each sample. These tracings were used for measurements of size and form, and for classification of the cells as will be described later.

The measurement of the rate of death in a culture and the determination of the limits of the death phase present some difficulties. In nearly all of the previous work the number of viable cells has been determined by agar plate counts, and the death phase has been defined as beginning at the point where the number of living cells starts to decrease. But undoubtedly the lethal factor begins to act much earlier than this, at least at the point where the growth curve shows an inflection from positive to negative acceleration, and probably earlier.

This has been demonstrated by Wilson,[5] who finds at all stages of growth a discrepancy between the microscopic count and the plate count which can only be explained by the fact that some of the cells are dying during the active growth period. By the time the culture has reached the beginning of the death phase as defined above there must be already a considerable number of dead or injured cells present, and this is clearly indicated in the observations here reported by the appearance in every case of stained cells (the significance of which is explained below) before there occurred any decrease in the plate counts. On the other hand, after the number of viable cells shows a decrease, and the culture may definitely be said to have entered the death phase, some of the cells are still multiplying, as indicated by the increase in the microscopic counts, which generally continues for some time longer.

[4] Proc. Soc. Exper. Biol. & Med., 1923, 20, p. 293.
[5] Wilson, G. S.: J. Bact., 1922, 7, p. 405.

In recent years another criterion of death in microorganisms has come into use, viz., the ability of the cells to absorb dyes. It has long been known that gram-positive organisms become gram-negative in old cultures, and R. and W. Albert[6] have shown that the loss of the gram-staining property in yeasts is correlated with the degree of autolysis in the dead cells. Fraser[7] studied the action of various dyes on living and dead yeast cells and found that these could be differentiated readily, especially by methylene blue. This procedure was used by Fulmer and Buchanan[8] to measure the death rate of yeast acted upon by disinfectants; they pointed out that the ability of the cells to absorb stains occurs after the cell has lost the ability to grow when transferred to a new medium. Burke[9] has shown that dead spores may be differentiated from living ones by staining with carbol fuchsin, and Koser and Mills[10] also found that the ability of the spores to absorb the dye was acquired later than the period when they lost the power to germinate. Seiffert[11] showed that dead bacteria will become stained with Congo red, and I have made use of this dye with a somewhat different technic[4] for differentiating living and dead cells.

It has generally been assumed that the staining of the dead cells is due to the increased permeability of the membrane, and this view would seem to be supported by the observation of Fraser[7] that dead yeast cells stain much more slowly with the less diffusible Congo red than they do with methylene blue. But neutral red will stain certain structures, the volutin vacuoles and granules, in living yeast cells, so that the membrane must be permeable to that dye; whereas dead cells are stained a uniform red in the same solution. And I have similarly found that Congo red will stain the metachromatic granules of diphtheroid bacilli and the sporogenous granules of spore-bearing bacilli at a stage of growth when certainly not more than a small proportion of the cells could be dead. It is therefore probable that the acquisition of the staining property in dead cells is due to a change in the adsorption properties of the protoplasm rather than to a change in the permeability of the membrane.

This change in the protoplasm probably occurs at a certain stage in the autolysis of the cells. This opinion is supported by the parallelism between the acquisition of the staining property in the unfixed cells and the loss of the gram-staining property of the fixed cells of yeasts, which has been shown to be due to autolysis; by the fact noted by all of the authors quoted above that the acquisition of the staining property occurs some time after the cells have lost their viability (with Congo red this is a period of some hours with bacteria killed by heat); and by the fact that dead cells, preserved in formalin, do not become stainable. With Congo red apparently a later stage of autolysis must be reached than with other dyes.

From the observations here reported three sets of data are available, the plate counts, the percentage of stained cells, and the microscopic counts (table 1 and fig. 2), from which the rate of death and dissolution of the cells may be computed. In figure 2 a, the values are all expressed on a percentile basis chosen as the form in which they could be most readily compared; the solid lines indicate the cumulative percentile

[6] Centralbl. f. Bakteriol., II, O., 1901, 7, p. 737.
[7] J. Phys. Chem., 1920, 24, p. 741.
[8] J. General Physiol., 1923, 6, p. 75.
[9] J. Infect. Dis., 1923, 32, p. 433.
[10] J. Bact., 1924, 10, p. 25.
[11] Centralbl. f. Bakteriol., I, O., 1922, 88, p. 151.

decrease from the maximum number of cells shown by the plate counts, the dashed lines the cumulative percentile increase in cells stained by Congo red, and the dot-and-dash lines the cumulative percentile decrease

TABLE 1

DATA ON GROWTH OF ESCHERICHIA COLI IN DIFFERENT MEDIUMS

Days of Growth	Plate Counts in Millions	Stained Cells, %	Microscopic Counts in Millions
	Alkaline		
1	2,790	0.0	2,555
2	3,650	3.7	3,820
3	3,200	5.1	4,760
5	2,265	8.8	5,350
7	1,700	11.7	5,500
10	760	12.8	5,760
14	54	20.1	5,670
19	8.7	23.6	5,600
25	1.61	56.7	5,380
	Neutral		
1	1,090	0.0	2,900
2	2,240	4.0	4,640
3	890	6.5	5,590
5	322.5	11.2	6,050
7	226	27.1	6,190
10	175	36.7	5,610
14	112.2	45.2	5,510
19	106	57.0	4,600
25	100.4	59.6	3,420
	Sodium Chloride		
1	1,540	1.5	1.320
2	1,660	3.1	1,772
3	1,750	10.3	1,957
5	645	15.8	1,858
7	230	21.1	1,611
10	24.1	45.9	1,410
14	7.6	62.8	1,240
20	1.12	82.0	1,075
	Acid		
1	2,370	2.2	2,630
2	2,675	8.8	3,200
3	3,400	13.5	3,026
5	4,700	32.6	4,650
7	1,160	39.4	5,011
10	325	47.8	6,616
14	85	58.2	5.420
19	44.8	71.1	5,160
25	18.2	81.8	4,095
	Calcium Chloride		
1	1,140	12.1	1,330
2	1,435	17.0	1,430
3	1,050	21.4	1,495
5	680	39.6	1,530
7	600	43.0	2,270
10	398	55.5	2,295
14	183	78.0	3,460
19	100	84.0	2,600
25	32.5	92.2	2,130

from the maximum number of cells shown by the microscopic counts. The plate counts show the time and rate at which the cells lose their ability to reproduce in a new medium; the staining of the cells occurs

at a later period and indicates a stage in their autolysis; the decrease in microscopic counts indicates the final dissolution of the cells. This being the case the curve for percentage increase in stained cells occupies in every case a position between the other two. If the rate of autolysis were constant in the different cultures, and at all stages in the same culture, the curves for plate counts and for stained cells should be parallel and the same distance apart. That this is not the case is apparent from figure 2[a]. While more accurate measures of this variation in the rate of autolysis may be obtained, it can be very simply expressed by the distance between the two curves on the 50 percentile line, i. e., by the time which elapses between the moment when 50% of the cells are dead and the moment that 50% of them are stainable. This was greatest in the alkaline culture (17 days), the other cultures in order of ascending autolysis rate being the neutral one (13.25 days), the NaCl culture (6.25 days), the acid one (4.25 days) and the one containing calcium chloride (4 days).

The highest rate of death was shown by the salt culture, the mean daily decrease in the plate counts being 21.8%; in the alkaline medium it was 16.5%, in the acid one 16.4%, in the calcium chloride agar 11.9%, and in the neutral culture 9.7%. The period of maximum death rate occurred in the neutral agar between the 2nd and 3rd days, the decrease per day being 60.2%; in the acid medium between the 5th and 7th days, 37.7%; in the medium with sodium chloride, 5th to 7th days, 32.2%; in the calcium chloride agar, 2nd to 3rd days, 26.8%; and in the alkaline culture, 10th to 14th day, 23.3%. The order of the cultures with regard to rate of autolysis does not agree with either of the above, and the rate of autolysis is therefore independent of the death rate.

The medium most favorable to growth, as measured by the maximum yield (plate counts), was the acid agar; the others, in descending order being the alkaline, the neutral, and those containing salt and calcium chloride. The death phase began earliest in the neutral and alkaline media, both showing the maximum plate counts on the 2nd day; in the salt agar and calcium chloride medium on the 3rd day; and in the acid agar on the 5th day.

The microscopic counts in the death phase are determined not only by the rates of death and autolysis, but also by the degree to which those cells still viable are multiplying; for the marked increase in the microscopic counts after the plate counts have reached their maximum can only be explained by the fact that some of the cells are still actively dividing and the progeny then soon dying. This occurred in greatest degree in the neutral medium, the maximum microscopic count showing

TABLE 2

PERCENTAGE OF CELLS OF DIFFERENT CLASSES IN VARIOUS MEDIUMS

Cell Class	Days of Growth 1	2	3	5	7	10	14	19	25	Mean
					Alkaline					
I	54.5	51.0	60.0	45.0	61.5	60.0	66.5	53.5	48.0	55.55
II	15.5	8.5	8.5	5.0	9.0	7.5	3.0	5.5	8.0	7.83
III	2.0	23.0	20.5	39.5	11.5	15.0	17.5	22.0	29.0	20.00
IV	26.5	9.5	4.0	2.0	11.5	13.0	6.5	4.5	3.5	9.00
V	1.5	1.0	0.5	2.0	2.0	1.0	1.0	4.5	3.5	1.89
VI	0.0	5.0	3.0	2.0	2.0	2.5	2.0	6.5	4.0	3.00
VII	0.0	2.0	4.0	4.5	2.5	1.0	3.5	2.5	3.0	2.55
VIII	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.05
IX	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.05
X	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.11
Index of variation	67.08	67.61	61.80	68.30	63.18	59.31	58.55	86.07	77.45	67.71
					Neutral					
I	42.0	48.5	45.0	57.5	53.5	52.5	45.5	46.0	44.5	48.33
II	11.5	12.5	16.0	11.0	25.0	15.5	10.0	13.0	11.5	14.00
III	40.0	15.5	17.5	19.5	10.5	15.5	18.0	17.5	26.0	20.00
IV	0.5	7.5	4.0	6.0	2.5	5.5	7.5	10.0	5.0	5.39
V	0.5	1.0	3.5	2.0	4.0	0.5	3.5	3.0	4.0	2.47
VI	1.5	11.0	8.0	3.0	1.0	5.5	9.0	5.5	5.5	5.55
VII	3.5	1.0	3.5	1.0	1.0	3.5	3.5	2.5	2.0	2.39
VIII	0.5	0.5	0.0	0.0	2.0	0.5	1.0	2.0	1.5	0.89
IX	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.05
X	0.0	2.0	2.5	0.0	0.5	1.0	2.0	0.5	0.0	0.90
Index of variation	64.37	87.82	115.27	55.98	73.17	86.03	119.25	95.41	80.70	87.56
					Sodium Chloride					
I	62.5	28.5	44.5	46.0	57.0	44.5	60.0	42.0	....	48.12
II	16.0	30.0	28.5	19.5	25.5	31.5	19.0	37.5	....	25.93
III	14.5	12.0	10.5	15.0	4.5	8.5	9.0	4.5	....	9.81
IV	2.5	0.0	1.0	3.0	1.0	3.5	6.0	11.0	....	3.50
V	2.0	5.0	4.0	7.5	4.0	3.5	4.0	2.5	....	4.06
VI	1.5	10.0	4.5	3.5	3.0	4.5	1.0	2.0	....	3.75
VII	0.0	1.0	0.5	2.0	0.5	0.0	0.5	0.0	....	0.56
VIII	1.0	11.0	4.0	3.0	4.5	3.0	0.5	0.0	....	3.37
IX	0.0	0.5	1.0	0.0	0.0	0.0	0.0	0.0	....	0.19
X	0.0	2.0	1.5	0.5	0.0	1.0	0.0	0.5	....	0.69
Index of variation	50.48	181.55	117.79	96.34	80.24	92.94	55.58	69.18	....	93.01
					Acid					
I	51.0	46.0	36.0	43.0	45.0	47.0	47.5	36.0	35.0	42.94
II	21.0	23.0	25.5	20.0	15.0	15.0	13.5	11.5	11.5	17.33
III	5.5	10.5	18.5	12.5	13.5	18.0	11.5	22.5	32.0	16.05
IV	14.0	12.5	11.0	10.0	13.0	8.0	10.5	15.0	3.0	10.77
V	3.0	2.5	1.5	7.0	2.5	5.0	7.5	6.0	3.5	4.50
VI	0.5	4.5	2.5	3.5	4.5	2.5	5.5	2.0	3.0	3.17
VII	3.0	0.0	4.0	2.5	4.0	2.0	2.5	5.5	12.0	3.94
VIII	2.0	0.5	0.5	1.0	1.0	1.0	1.0	0.5	0.0	0.83
IX	0.0	0.5	0.0	0.5	1.0	0.5	0.0	0.5	0.0	0.33
X	0.0	0.0	0.5	0.0	0.5	1.0	0.5	0.5	0.0	0.33
Index of variation	82.73	75.10	88.72	91.12	106.37	95.69	95.81	109.76	85.73	93.42
					Calcium Chloride					
I	48.0	38.0	42.0	41.5	42.0	32.0	39.0	35.0	42.0	39.94
II	23.0	23.5	23.5	21.0	25.0	8.0	17.5	10.0	10.0	17.94
III	7.0	6.5	5.0	11.0	8.0	24.5	8.0	24.5	24.0	13.17
IV	1.0	5.0	7.0	1.5	1.5	2.0	4.0	3.0	2.0	3.00
V	10.5	14.5	9.5	13.5	11.5	11.0	9.0	8.5	6.5	10.50
VI	7.5	4.5	9.0	6.5	3.0	5.0	6.0	4.5	5.5	5.72
VII	2.5	5.0	0.5	1.5	1.0	3.5	3.0	3.5	2.0	2.50
VIII	0.0	2.0	0.5	1.5	1.0	4.5	4.0	2.5	3.5	2.17
IX	0.0	0.5	0.5	1.0	5.5	8.5	8.0	6.5	3.5	3.78
X	0.5	0.5	2.5	1.0	1.5	1.0	1.5	2.0	1.0	1.29
Index of variation	88.06	123.78	127.97	118.30	161.35	226.02	220.21	200.83	148.63	157.25
Mean index of variation.....	70.54	107.19	102.35	86.01	96.86	111.99	109.88	112.35	98.13	

an increase of 176% over the maximum plate count. In the calcium chloride medium this increase was 141%, in the alkaline culture 58%, in the acid 41%, and in the salt agar 12%. It is interesting in this connection that the first two cultures named also had a high incidence of constricted (dividing) cells (table 2).

It is difficult to obtain a satisfactory measure of variation in the form of the cells. The area-length index, i. e., the area of the projected image divided by the length squared, serves admirably as long as the cells are symmetrical, and if elongated, are of uniform diameter, and has satisfactorily been applied to a study of variations in form in young cultures.[12] But, as has been shown previously,[3] it does not serve to classify those cells which are most striking in the death phase; cells with bulgings and constrictions, buds and branches. The procedure described below is admittedly not entirely satisfactory, being rather artificial and involving to a certain extent the personal equation, but in the absence of anything better it may serve to supply at least an approximate quantitative expression of the degree of variation in the form of the cells.

The cells have been arbitrarily divided into 10 classes, and classified by simple inspection. The percentage frequency of all the classes in all of the cultures at all stages of growth was determined, and each class was then given a value, the reciprocal of this frequency. Then in each sample the percentage frequency of each class was multiplied by this value for the class, and the mean of the products taken as a measure of the degree of variation. This measure, termed the index of variation, expresses the degree of variation in each sample in terms of the degree of variation in the entire series of 5 cultures. Samples having more than the average number of the more frequent cell types will have an index of less than 100; samples having more than the average number of the less frequent cell types will have an index greater than 100.

The 10 classes are described below and illustrated in figure 1. This illustration was prepared by selecting a number of drawings from each class of all of the cultures, more or less at random, and tracing them. They are therefore fairly accurate reproductions of the cells and serve to indicate the variety of cell forms encountered. The classes are numbered in the order of their mean frequency.

Class I. Straight cells of uniform diameter having area-length indexes from 21 to 40, inclusive.

Class II. All cells of uniform diameter having a curved axis. As has been shown in the case of the cholera vibrio,[3] there is a correlation between the curvature and the degree of slenderness of the cells, and this is apparently also true of the colon bacillus, the majority of the cells in this class having a low area-length index.

Class III. Straight cells having an area-length index from 41 to 60, inclusive.

Class IV. Straight cells having an area-length index less than 21.

Class V. Cells showing a greater thickness at one end than the other, but not including cells having a swollen portion at one end separated from the rest of the cell by a constriction, which were placed in Class X.

Class VI. Cells with a central constriction, as though about to divide.

Class VII. Spherical cells, i. e., symmetrical cells having an area-length index greater than 60.

Class VIII. Cells with lateral projections, either as small buds or as definite branches.

Class IX. Cells showing a central bulging.

Class X. Cells with terminal buds, i. e., showing a constriction near one end, or with a small nipple like projection at one end.

[12] Henrici, A T.: Proc. Soc. for Exp. Biol. and Med., 1923, 21, 215.

It will be seen that no great degree of judgment is required in placing the cells in these various classes save in the border zones between the 4 classes of symmetrical cells; spheres, oval forms, normal rod forms, and slender filamentous forms. These were divided as indicated above by the

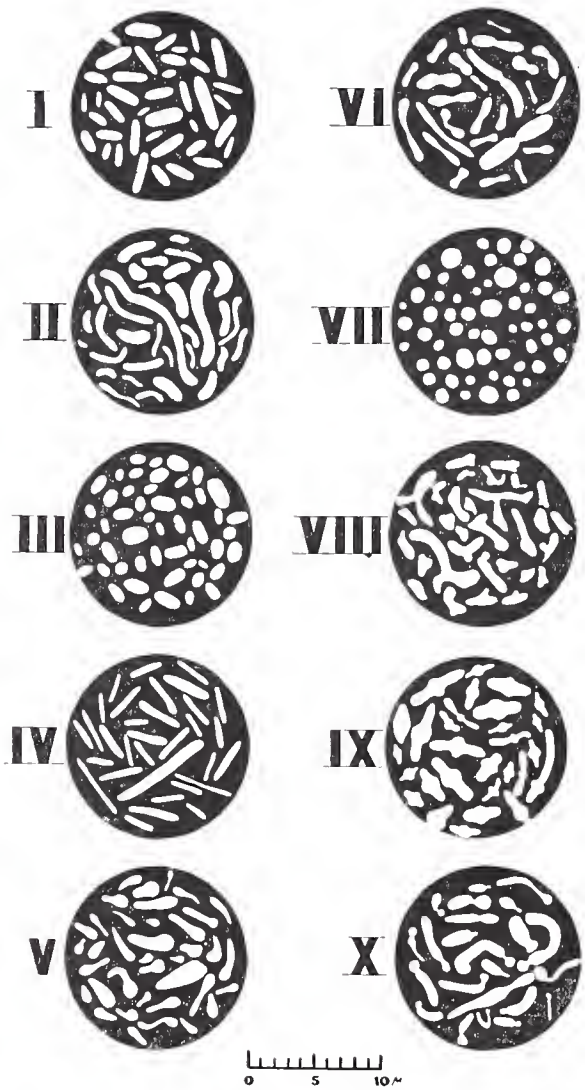

Fig. 1.—Ten classes of cell forms of Escherichia coli in order of frequency (p. 435).

area-length index. Occasional cells were encountered which presented characters of more than one class, as for instance curved cells with terminal buds. They were placed according to which character seemed most prominent.

The incidence of these classes in the 5 cultures at various stages of growth is given in table 2 together with the indexes of variation computed from them. The mean frequencies of the 5 cultures at all stages of growth are shown graphically in figure 2[b], the numbers inserted in the graphs being the means indexes of variation.

The fluctuations of the index of variation day by day are somewhat irregular and do not appear to be highly significant; this is perhaps

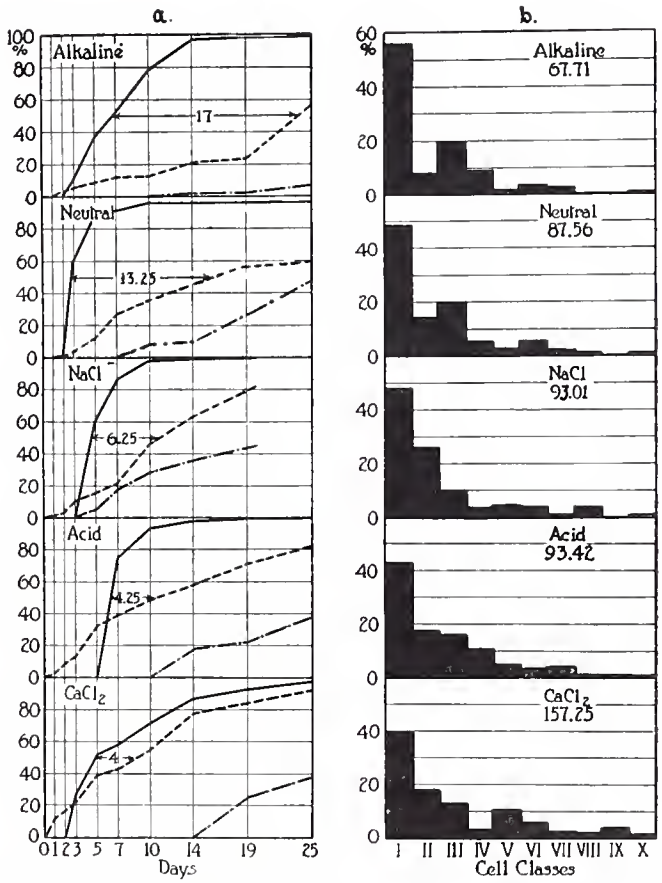

Fig. 2.—a. The cumulative percentile decrease from the maximum number of cells: by plate count—solid line; by Congo red stain—dash; by microscopic count—dot and dash. The rate of autolysis is indicated by the distance at the 50% level. b. The frequencies of the cell classes.

largely due to the small number of cells in each sample. The alkaline culture showed very little variation; there was a slight rise on the 19th day followed by a decline, corresponding with the period of decrease in death rate. In the neutral medium there occurred a well defined increase reaching the maximum on the 3rd day, corresponding with the beginning

of the death phase; this was followed by a decrease and a second increase with its maximum on the 14th day, at which point the death phase had nearly reached its end, the number of viable cells remaining nearly the same during the remaining period of observation. The medium containing NaCl showed a very pronounced rise in the index of variation on the 2nd day, due largely to the remarkably high incidence of budding and branching cells, followed by an irregular decline. The acid culture exhibited two maximums, one on the 7th day corresponding with the beginning of the death phase, and one on the 19th day when the death rate was decreasing. The medium containing $CaCl_2$ gave a slight initial rise followed by a decline, then a pronounced increase which was sustained until the 19th day, there being a marked reduction in the variation of the cells on the 25th day.

These fluctuations with time, while somewhat irregular and varying considerably in the different cultures, show in general a tendency to the formation of two maximums, one corresponding with the beginning of the death phase and one with the end. By computing the mean index of variation of the 5 cultures for each day we increase the number of cases and obtain a correspondingly more accurate picture of the variations with time. There occurs a sharp rise at the beginning of the death phase followed by a decrease, then a second more sustained rise with another marked decrease on the last day. This final drop in the degree of variation in the form of the cells, occurring during the period of decreasing death rate as measured by the plate counts and of increasing rate as measured by the microscopic counts, can readily be explained by the final dissolution of those cells which presented the greatest departure from the "normal" form, and may be considered to support the argument that these irregular cells are actually dead. I am at a loss, however, to explain the initial rise and fall in the index of variation.

It is not possible to establish any correlation between the degree of variation in form and the rate of death. In order of magnitude of their indexes of variation the 5 cultures are arranged as follows: alkaline (lowest), neutral, NaCl, acid, $CaCl_2$.

It will be noted that the values for the salt and acid cultures are nearly identical. But the figures for the salt cultures are not directly comparable with the others since no observation was made on the 25th day. Since in all of the other cultures there was observed a decrease in variation on the last day, it is probable that this would also have occurred in the medium with sodium chloride, and that the mean index of variation would have been reduced.

The rank order of the cultures with regard to their index of variation does not correspond with any of those given above for the rate of death or the favorableness of the medium to growth. In fact, the cultures containing calcium chloride, which gave quite the highest degree of variation in form had nearly the lowest death rate.

This rank order does, however, correspond definitely with the rank order of the cultures according to their rate of autolysis as has been indicated above, and while the correlation is not a perfect one, considering the imperfect nature of the measures both of rate of autolysis and of variation in form, it is close enough to be significant.

This correlation between the form of the cells and their autolysis in the death phase offers a clue as to the real nature of the involution forms. The "normal" types of cells, occurring almost to the exclusion of all others in the resting phase, are comprised in classes I to IV, i. e., slender to plump rods, some being curved. These forms, it may be postulated, are maintained by a certain degree of rigidity in the cell membrane. If autolysis be delayed, even though the death rate is relatively high (as in the alkaline culture) these forms will be maintained during the death phase. But if beginning disintegration leads to a softening of the cell membrane, the force of surface tension will lead to an alteration in form. If the softening be first localized at a point on the side of the cell it will cause a local bulging leading to budding and branching forms as in class VIII; if terminal it will give rise to such cells as are found in classes V and X, depending on the size of the area involved; if involving a girdling area around the cell it will produce such forms as are found in class IX. If the softening involves the entire cell it will give rise to oval and eventually spherical types (III and VII).

While the differentiation between stained and unstained cells is quite easily made by inspection through the microscope, there is not sufficient contrast in the photographic negative when projected at the magnification used to certainly separate the two classes when making the tracings. Consequently no comparison can be made of the variation in form between the stained and unstained cells. Microscopic observation indicates, however, that the majority of the unusual forms, as budding, branching and bulging cells, are not stained. This, however, does not necessarily indicate that the variation in form is not due to the same process which leads to the cells becoming stainable; the changes in the cell membrane may occur in an earlier stage of the process.

SUMMARY

Measures of the rate of death and of autolysis, and of variation in form of bacteria are described and discussed. The rate of autolysis may be estimated by the time intervals between the curves for death of the cells as determined by plate counts, and as determined by their staining with Congo red. The degree of variation in form has been computed by the products of the frequency of the various cell types and the reciprocal of their mean frequency, the fluctuations being expressed as index numbers.

The rate of death and autolysis, and the degree of variation in form have been determined in 5 cultures of Escherichia coli grown on peptone agar; acid, neutral and alkaline in reaction, and containing sodium chloride and calcium chloride respectively.

No correlation could be established between the degree of variation in form and the rate of death or the favorableness of the medium for growth.

There was found a definite rank order correlation between the degree of variation in form of the cells and the rate of autolysis of the dead cells. The rate of autolysis in the various cultures was as follows: alkaline, 17 days; neutral, 13.25; salt, 6.25; acid, 4.25; calcium chloride, 4. The mean index of variation in form of the cells was as follows: alkaline, 67.71; neutral, 87.56; NaCl, 93.01; acid, 93.42; $CaCl_2$, 157.25.

STUDIES ON EXPERIMENTAL ENCEPHALITIS

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Since 1919 there have been several reports purporting to establish the etiology of epidemic encephalitis. The organism described by Loewe and Strauss [1] as the cause of this disease has not borne the test of time and subsequent experimentation. The same may be said of the streptococcus described by Rosenow.[2] The problem of studying this disease in rabbits has been complicated by the appearance of spontaneous encephalitis in these animals which was first described by Bull.[3] In recent years, however, this objection has been largely obviated by the work of Wright and Craighead,[4] Doerr and Zdansky,[5] Levaditi and Nicolou and Schoen,[6] Cowdry and Nicholson,[7] Goodpasture,[8] McCartney [9] and others who have described the microorganism causing this disease.

Out of 372 normal rabbits McCartney found as high as 76% of the animals affected in some groups. The most common lesion histologically consists of focal areas of necrosis near the blood vessels, perivascular infiltration and a layer of mononuclear cells around the focal areas. There are apparently great variations in the lesions and occasionally small areas of epithelial-like cells without mononuclears are found scattered throughout the brain. The histologic lesions in this disease taken together with the clinical course of the disease serves to differentiate it from other forms of encephalitis in rabbits.

We have been concerned especially with the form of encephalitis produced in rabbits with the herpes virus. Levaditi and Harvier [10] in 1922 described a form of encephalitis produced in rabbits by an ultramicroscopic virus obtained from a case of encephalitis. Much criticism has been directed against this virus as having anything to do with epidemic encephalitis. Flexner [11] has pointed out that out of thirty sets of specimens from cases of epidemic encephalitis Levaditi succeeded in

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[1] J. Am. M. A., 1919, 73, p. 1056.
[2] J. Infect. Dis., 1924, 34, p. 329.
[3] J. Exper. Med., 1917, 25, p. 557.
[4] Ibid., 1922, 36, p. 135.
[5] Schweiz. med. Wchnschr., 1924, 54, p. 150.
[6] Compt. rend. Soc. de biol., 1923, 89, p. 984.
[7] J. Am. M. A., 1924, 82, p. 545.
[8] J. Infect. Dis., 1924, 34, p. 429.
[9] Brit. M. J., 1924, 2, p. 1159.
[10] Ann. de l'Inst. Pasteur, 1920, 34, p. 954.
[11] J. Am. M. A., 1923, 81, pp. 1688, 1785.

establishing in rabbits only one active virus and so represents really a single unequivocal experimental result. Numerous strains of herpes virus associated with this disease have been isolated in various parts of the world. Among the well known strains may be included the Basel strains isolated from the spinal fluid and brain substances from cases of encephalitis by Doerr and Schnabel and Doerr and Berger; the Berlin strain isolated by Schnabel from an acute case of this disease; the Wien strain isolated by Luger and Lauda; (the virus isolated by Davide, Kling and Liljenquist in Sweden was probably the spontaneous form); the virus of Bessemans and von Baeckel and others. Lipschutz [12] has described the viruses obtained from the vesicular contents of different forms of herpes and more recently has obtained them from herpes zoster. Cole and Kuttner,[13] however, in a series of nine cases of herpes zoster were unable to isolate a single strain which suggests that the herpes virus, when found in these lesions, is coincidental and bears no relation to the etiology of the disease. A description of these various strains and an excellent summary of the work in this field is contained in a review by DaFano.[14]

It is apparently agreed by all investigators that a form of encephalitis can be uniformly produced in rabbits with the herpes strains and cross immunity experiments with most of these viruses, including the Levaditi strain and the Beckley strain of Flexner and Amoss, seem to identify them all with the herpes family. That some of these strains may be isolated from the spinal fluid or brain substance from cases of epidemic encephalitis may or may not be significant. Inasmuch as no other organism has been found in these cases in as great a number of instances we feel that their significance should not be discounted. Perdrau [15] has recently described three viruses which he isolated from the brain material of three cases of encephalitis which he believes are similar to a neurotropic strain of herpes virus which he had previously described as coming from a case of herpes labialis. He states that such viruses have a selective action; that they are dermatropic or neurotropic and that the neurotropic virus introduced intradermally into rabbits produces a fully established immunity in eleven days which lasts for about three months.

A great deal of attention has been given to the occurrence of minute intranuclear bodies in this form of encephalitis, Lipschütz [16] has described these bodies in the vesicular lesions and Goodpasture [17] and von Glahn

[12] Arch. f. Dermatol. u. Syph., 1921, 136, p. 428.
[13] J. Exper. Med., 1925, 42, 799-819.
[14] M. Sc., 1924, 10, p. 355.
[15] Brit. J. Exper. Path., 1925, 6, p. 41.
[16] Centralbl. f. Bakteriol., O. I., 1921, 87, p. 303.
[17] Am. J. Path., 1925, 1, p. 1.

and Pappenheimer [18] and others have described in detail the intranuclear inclusions in the nerve substance. Goodpasture and Teague [19] have demonstrated in beautiful experiments that the herpes virus passes up the axis cylinder of the nerve from its point of entrance to the central nervous system. The significance of these intranuclear bodies is still undertermined though it is possible, as v. Prowazek [20] has suggested, that they represented the virus granules proper which are surrounded by the reaction products of the invaded cell.

In 1924-25 one of us began a study of experimental encephalitis with LeFèvre [21] in Brussels. These experiments which served as a starting point for the later work are here reviewed briefly.

The LeFèvre herpes strain isolated from a case of herpes genitalis and the so called Levaditi encephalitis virus were used. Emulsions of rabbit brains containing either strain, incubated with fresh serum from ten days to two weeks, displayed an attenuation of the virus. In 1 : 1,000 dilutions of the original emulsion in fresh rabbit serum, attenuation was brought about in as short a time as seven days at incubator temperature. Similarly if emulsions were prepared in salt solution and dilutions of 1 : 100,000 were prepared with the herpes strain and 1 : 10,000 with the Levaditi virus it was found that both viruses became attenuated slightly within three days and more markedly after ten days when left at ice box temperature.

For example: 0.2 cc. of the herpes emulsions in salt solutions of 1: 100,000 and 1: 10,000 injected subdurally into rabbits when the emulsions were first prepared, produced a typical encephalitis and killed the animals within three days. After three days in the ice box the 1: 100,000 dilution failed to produce encephalitis and the animal remained normal several weeks after the injection. The 1: 10,000 dilution, in the same dose, required five days to produce symptoms, and the animal died the same day, showing a slight attenuation of the virus. After ten days in the ice box the 1: 10,000 dilution required seven days to produce symptoms and death, while the animal which received the 1: 100,000 dilution survived twenty-nine days. After thirty days in the ice box hoth dilutions were found to be innocuous. Essentially the same results were obtained with the Levaditi virus in salt solution emulsion diluted in 1: 10,000 and 1: 1,000. This virus is not as virulent as our herpes strain and when first tested in the above dilutions required six and five days, respectively, to kill. After three days at ice box temperature they required eight and six days to kill and after ten days the 1: 1,000 dilution killed in six days while the animal receiving 1: 10,000 dilution survived the infection. After thirty days in the ice hox both dilutions of the Levaditi virus were found, as in the case of herpes, to be innocuous.

It was thought possible by employing such emulsions of these viruses, attenuated in salt solution at ice box temperature or in fresh rabbit serum at incubator temperature, that immunity could be produced by several subdural injections by gradually increasing the strength of the emulsion. Two groups of rabbits were accordingly put under treatment using first the 1 : 100,000 dilution of herpes and the 1 : 10,000 dilution of the Levaditi virus. The animals received weekly injections over a period of three months gradually increasing the strength of the emulsion to a

[18] Am. J. Path., 1925, 1, p. 445.
[19] J. M. Research, 1923, 44, p. 121.
[20] Arch. f. Protistenk., 1907, 10.
[21] LeFèvre, M., and McKinley, E. B.: Unpublished data.

1 : 50 dilution. At this point we lost the whole series showing that it is impossible to immunize rabbits through the nervous system, at least by this method.

Further experiments were carried on in trying to culture both of these strains. Since none of our procedures gave any degree of success we will not go into detail of the experiments but, for the reasons heretofore stated, simply mention them as a matter of record. Attempts to cultivate these viruses in Noguchi's medium at various reactions of P_H ranging from 6.2 to 7.6 demonstrated clearly that these viruses do not easily survive in such mediums, let alone multiply. This is in accordance with the work reported by other investigators. Experiments to demonstrate the passage of these viruses from the cut surface of an infected brain into a normal fresh brain when placed in close apposition to its cut surface, under anaerobic or aerobic conditions, were failures. The virus could be recovered from the cut surface of the fresh brain but not from the depths of the tissue, showing that it was merely contact contamination. These experiments were done at various temperatures with negative results. It was found that freezing at —16 C. for as long as 24 hours did not affect the virulence of either of these strains. It was further observed that the injection subdurally of other foreign materials such as suspensions of red blood cells, white blood corpuscles, fresh emulsions of rabbit testicle, fresh rabbit brain, and blood stroma proved innocuous. In no case were we able to produce any effect upon the animal by such inoculations.

During the past year we have continued the study of these two strains begun in 1924. Our herpes strain brought from Brussels (four months in glycerol) was found to be slightly attenuated. The Levaditi virus under the same conditions was found to have lost its infectivity entirely. We are indebted to Dr. Flexner for furnishing us with his Levaditi strain 926. Our herpes strain, after three passages, quickly regained its old virulence and produced encephalitis and death of the animal within three or four days. The Levaditi strain obtained from Dr. Flexner kills on the average in about six days. In this respect it acts like the Brussels strain.

From the work of others and our own experiments it is well known that a typical encephalitis usually resulting in death of the animal within a few days, can be produced in rabbits by various routes. We have found this to be true by either intravenous, intradermal, intracorneal, intraperitoneal and subdural inoculations. We were interested to confirm more definitely the close biological relationship existing between these two viruses and consequently chose the subcutaneous route for our injections and employed emulsions in fresh rabbit serum which former experience had taught would attenuate the viruses.

Mixtures of normal serum and infected brain material were prepared of each virus in several dilutions (concentrated, 1 : 100, 1 : 1,000 and 1 : 10,000) and placed in the ice box for preservation. It will be noted in table 1 that the concentrated emulsions (1 Gm. infected brain to 2 cc. fresh serum) became definitely attenuated

after 30 days at ice box temperature. The 1 : 100 dilutions are attenuated after 20 days at this temperature while the 1 : 1,000 and 1 : 10,000 dilutions are markedly attenuated after seven days under the same conditions. Cross immunity experiments were commenced on Dec. 12, 1925, with a small series of animals. The animals received ten injections of 0.5 cc. and 1 cc. subcutaneously over a period of three months. On Feb. 2, 1926, the herpes vaccinated animals were tested with the Levaditi virus by subdural inoculation and the Levaditi virus vaccinated animals were tested with the herpes virus by subdural injection. In table 2 it is apparent that we were able to produce a high grade of immunity for the herpes strain in the series treated with a Levaditi serum-virus mixture. All of this series survived the subdural injection of a concentrated emulsion (in salt solution) which

TABLE 1

INOCULATION OF RABBITS WITH INFECTED BRAIN EMULSIONS IN FRESH RABBIT SERUM

Death from typical encephalitis (+), and survival (0) of inoculated rabbits.

Days in Icebox	Concentrated (1 Gm. Brain + 2 Cc. Serum)	Dilutions of Brain Emulsion Supernatant		
		1:100	1:1000	1:10,000
3	+	+	+	+
7	+	+	0	0
10	+	+	0	0
20	+	0	0	0
30	(+) delayed symptoms and death	0	0	0

TABLE 2

CROSS IMMUNITY TESTS IN RABBITS WITH HERPES AND LEVADITI ENCEPHALITIS VIRUSES

Rabbit Number	Number of Subcutaneous Injections with Serovaccine 12/12/25 to 2/9/26	Date Virus Subdurally	Date of Death	Number of Days Survival
1	10 herpes virus	Levaditi 2/24/26	3/ 1/26	5
2	10 herpes virus	Levaditi 2/24/26	3/18/26	22
3	10 herpes virus	1/19/26	Died from other infection	
4	10 Lavaditi virus	Herpes 2/24/26	.....	61 (4/26/26)
5	10 Levaditi virus	Herpes 2/24/26	.....	61 (4/26/26)
6	10 Levaditi virus	Herpes 2/24/26	.....	61 (4/26/26)
7	0, control for herpes virus	Herpes 2/23/26	2/26	3
8	0, control for Levaditi virus	Levaditi 2/24/26	3/ 3	7

produced a typical encephalitis and death in the control within three days. One of the animals which received herpes serovaccine was lost during the course of the experiment due to an intercurrent infection, and another when tested with the Levaditi virus, died within five days, showing no immunity to this strain. A third animal proved to be well protected against the Levaditi virus. Though this animal died 22 days after receiving the subdural injection it showed no symptoms of encephalitis and had a bad diarrhea and snuffles. When it was noticed that this animal was not in good condition and would probably die, a quantity of blood was taken from it, the serum of which was tested later for its neutralizing effect upon both the herpes and the Levaditi virus.

A preliminary experiment was made to test the neutralizing titer of the serum from those animals which had survived the cross immunity experiments for both the herpes virus and the Levaditi strain. It will be seen in table 3 that the serum from rabbit 5 which had been immunized with the Levaditi serum-virus mixture possessed definite neutralization properties. One cc. of the supernatant fluid,

after centrifugalization of a concentrated emulsion in salt solution, was added to 1 cc. of the antivirus serum. After one week in the ice box 0.4 cc. of the mixture was injected subdurally into the test animal. The animal which received 0.4 cc. of brain emulsion Levaditi virus plus antiserum from rabbit 5 survived the injection over two months while the control animal which received the same brain emulsion plus fresh rabbit serum after one week in the ice box died on the fifth day. The serums from the other two Levaditi virus immune animals were not so effective against the virus as the serum from rabbit 5. The test animal for antiserum of rabbit 4 died in nine days following the subdural injection which is a delay over the control animal but is not in itself very significant. The test animal for rabbit 6 antiserum died in six days showing no delay whatever. Serum from rabbit 2, a herpes immune animal, showed no neutralizing effect for our herpes strain in the test animal.

TABLE 3

Tests of Serums, of Surviving Inoculated Rabbits, with Viruses (Brain Emulsion Supernatant)

Rabbit Number	Mixtures Virus; and Serum 1 Cc. Each	Inoculation of Rabbits Subdurally with 0.4 Cc. Mixture after 7 Days Contact, 3/24/26	
		Death Date	Days Survival
9	Levaditi virus emulsion; rabbit 4 immune serum	4/ 2/26	9
10	Levaditi virus emulsion; rabbit 5 immune serum	.....	69 living (6/1/26)
11	Levaditi virus emulsion; rabbit 6 immune serum	3/30/26	6
12	Herpes virus emulsion; rabbit 2 immune serum	3/28/26	4
13	Levaditi virus emulsion; fresh rabbit serum	3/29/26	5
14	Herpes virus emulsion; fresh rabbit serum	3/28/26	4

From these four immune animals, then, one serum was found to possess definite virucidal properties. The serum from this rabbit 5 has been studied in some detail both for its virucidal effect upon the Levaditi strain and for our herpes strain.

In the first part of table 4 it will be seen that this serum in as short a time as 24 hours exerts a definite virucidal effect upon the Levaditi strain in that there is a delay in the onset of symptoms and death in the test animal of 16 days. The control animal with normal serum died in seven days. Apparently the virucidal effect of the serum manifests itself within the first 3 or 4 days. After that there is a definite delay in the death of the test animal but apparently no greater protection is brought about. In this experiment rabbit 17 receiving antiserum plus virus after 4 days in contact at ice box temperature, showed no effects following the injection while the control died in 7 days. This of course suggests the question of the individual resistance of the animal which must necessarily be a factor. Too much emphasis must not be given, however, to the possibility of individual resistance in this experimental disease since we have found that practically 100% of rabbits succumb with typical symptoms following the subdural injection of either of these viruses. As has been mentioned above the herpes strain is exceedingly active even when diluted 1 : 100,000 and the Levaditi virus is very virulent in dilutions of 1 : 10,000. Consequently in the tests we have made to determine the virucidal effect of immune serums, our results are even more significant since the test animal received concentrated virus.

In another experiment we have tested the virucidal effect of immune serum for our herpes strain. In the second part of table 4 it appears that contact of the herpes virus with the immune serum for 24 hours was sufficient for attenuation. Rabbit 22 died in nine days following the subdural injection of 0.4 cc. of the virus-antiserum mixture. Rabbits 23, 24 and 25 which received mixtures of virus and antiserum after 72 hours, 120 hours and 168 hours in contact have survived the injection to the time of writing, which is 39, 37 and 35 days respectively. The control virus emulsion killed within four days and the control virus-fresh serum mixture after 168 hours in contact in the ice box killed within four days.

Since the preparation of this paper the work of Zinsser and Tang [22] with herpes virus has appeared. Zinsser and Tang found that serums from actively immune rabbits, immunized by corneal inoculation with the herpes virus, possess definite neutralizing properties for the virus when left in contact a few hours in the incubator. These authors comment upon the irregular results which have been reported by various investigators in this matter and suggest that these differences are not due to differences in serums but perhaps to the ordinary technic of using brain material for virus when some of the virus is intracellular and some extracellular, and the serum may be blocked from contact with the intracellular fraction. It will be recalled that in our experiments we have used supernatants—not dilutions of emulsions—and we find that the serums from some of our animals seem to possess no neutralizing properties. Also our animals have been immunized in an entirely different manner from those heretofore reported and in so far as we have been able to determine our serum-virus mixtures have produced no reactions which would indicate the presence of any disease process. Like Zinsser and Tang, we have found that our rabbits which survive neutralized serum-virus mixtures do not possess any immunity to fresh virus.

TABLE 4

EFFECT OF THE APPARENTLY IMMUNE SERUM OF RABBIT 5 ON THE LEVADITI-ENCEPHALITIS AND HERPES VIRUSES

Rabbit Number	Mixtures *		Days of Contact	Days Survival after Subdural Inoculation, 0.4 Cc. Mixture
	Virus	Serum		
15		—	—	4 (Received 0.2 cc. virus without serum)
16	Levaditi, encephalitic brain emulsion	Immune	1	16
17		Immune	4	56 (Living 6/28/26)
18		Immune	5	10
19		Immune	7	17
20		Fresh, normal rabbit	7	7
21		—	—	4 (Received 0.2 cc. virus without serum)
22	Herpes, brain emulsion	Immune	1	9
23		Immune	3	39 (Living 6/28/26)
24		Immune	5	37 (Living 6/28/26)
25		Immune	7	35 (Living 6/28/26)
26		Fresh, normal rabbit	7	4

* Levaditi virus and serum, 1 cc. each; herpes virus and serum, 0.5 cc. each.

It seems apparent from these experiments that a potent antivirus serum for the herpes virus and the Levaditi virus can be obtained in rabbits after hyperimmunization with serum-virus mixture subcutaneously over a long period of time. To our mind this seems additional evidence that the Levaditi virus is in reality a herpes strain. Taken together with the cross immunity experiments of other workers as well as our own there seems to be no doubt of this fact. In subsequent experiments we shall refer to this virus as herpes virus 926.

[22] J. Exper. Med., 1926, 44, p. 21.

In a recent communication[23] we have reported the effect of ultraviolet light upon our herpes strain and herpes virus 926. Exposure of concentrated emulsions of these viruses in quartz tubes at a distance of one foot for 20 to 40 minutes was sufficient to destroy them. The animals used in these experiments were later tested for immunity but in every instance the animals succumbed to subdural injections of unrayed virus. Further experiment has shown that as short a time as five minutes is sufficnent to destroy the herpes virus when exposed to ultraviolet light under these conditions.

Perdrau[15] has described certain skin reactions in rabbits obtained when the herpes virus is rubbed gently into the scarified skin. The reactions appear within 24 hours and if not followed by symptoms of encephalitis are said to clear up within a few days and the animal develops a full immunity against the subdural injection of the virus in about eleven days. We have attempted to repeat these experiments but have had little success when we employed emulsions of the virus in salt solution. Our animals have not died from such superficial inoculation but neither have they exhibited any skin reactions. When we have used emulsions of the virus in fresh rabbit serum we have obtained, in some cases, most intense reactions which have persisted for a week or more. These animals were tested by subdural injection for immunity between the 11th and 15th days and died in every case within three or four days following the inoculation. It seemed apparent that no immunity was produced in these animals as a result of one injection intradermally of a virus which, to all intents and purposes, had in the past demonstrated what might be termed neutrophic properties.

It has been found possible to study the brains of rabbits infected with the Levaditi encephalitis and herpes viruses very early in the course of the disease.[24] For this study we have given rabbits intracerebral injections. Twenty-four hours after the injection the trephine opening in the skull has been bored through and the cap of bone removed. This opening can be further enlarged and the hemorrhage controlled with bone wax. With the aid of a binocular microscope giving stereoscopic vision, supported upon an especially constructed mechanical stage, it is possible to study in detail the cerebral cortex. In as short a time as 24 hours after injection there is found a definite hyperemia of the cortex. The blood vessels stand out and the circulation can be seen and followed. This is greatly facilitated by the injection of India ink (one part ink to two parts salt solution). There is also noted a marked widening of the perivascular lymph spaces which is not found in normal rabbit brains. Indeed, it is impossible to see or follow the circulation in the brains of uninfected rabbits. This procedure has made it possible to study the effect of various injections of salt solutions and many drugs and glandular products upon the circulation in the cortex under these conditions. A full account of this work will be given in a separate publication.

In the course of our experiments we have had access to only three cases of herpes zoster. Vesicular fluid was taken from each of these cases and injected subdurally into rabbits. Our results have been uniformly negative with this material. In no case have our animals shown any effects following the injection. This is in accordance with the findings of Cole and Kuttner, though we hesitate to draw any conclusions

[23] McKinley, E. B.; Fisher, R., and Holden, M.: Proc. Soc. Exper. Biol. & Med., 1926, 23, 408-12.

[24] Tilney, F.; Howe, H. S., and McKinley, E. B.: In press.

from such a small number of cases. It seems probable, however, that the interpretation placed upon the finding of herpes virus in the lesions of herpes zoster by Cole and Kuttner is the correct one.

DISCUSSION

From the cross immunity experiments we have described above there seems little doubt that our herpes strain isolated from a case of herpes genitalis is biologically similar to the herpes strain 926. There are, however, several points of difference between these two viruses. We have been impressed with the uniform virulence of our herpes strain and as previously stated we have found that .2 cc. of a brain emulsion containing this virus is sufficient to produce typical symptoms and death in rabbits within three or four days. In the case of herpes virus 926 the average time required to produce symptoms and death is six days. These averages have been determined on about 75 passages with each virus. Furthermore our herpes strain appears to produce a marked congestion in the brains of the infected animals while the herpes 926 practically never does this. Even during so large a number of passages the herpes 926 has never acquired a greater fixed virulence. When LeFèvre first isolated our herpes strain from herpes vesicles it required 66 days to kill the first animal and then by repeated passage its virulence was gradually increased until it reached a fixed virulence of three days. The symptoms produced in rabbits are identical with both viruses, though the animals infected with our herpes strain succumb more rapidly after the onset of symptoms than do those infected with herpes strain 926. In other words, the disease produced is definitely more acute with the LeFèvre virus.

In these experiments we have studied a purely experimental form of encephalitis which is produced in rabbits by the herpes virus. While it is highly desirable to determine the etiology of true lethargic encephalitis in human beings we have thought that additional data concerning these known viruses, which produce in many respects a disease in animals which is much like encephalitis in human beings, and which have in several instances been isolated from the spinal fluids or brain material from human cases, was highly important. The possibilities regarding the etiology of lethargic encephalitis are that the etiological agent of lethargic encephalitis has not as yet been found, and that the herpes virus is the cause of this disease. In this case, as Perdrau has pointed out, the virus is recovered from those cases in which there exists no immunity whatever, the patient dies and the virus is found in the spinal fluid or brain tissue. In other cases where there is a natural immunity

the patient remains immune, where there is a partial immunity established the patient may recover or remain with a chronic form of the disease. In such cases the virus cannot be recovered. On the other hand there is a good deal of evidence to show that the herpes virus bears no relation whatever to this disease. At the same time there is just doubt in the matter either way and further investigation will be necessary to establish the relation of the herpes virus to the etiology of encephalitis or to discredit it.

CONCLUSIONS

Two strains of herpes virus (one Levaditi virus) have been found to become attenuated when dilutions are made in physiologic salt solution or in fresh rabbit serum and kept at ice box temperature or incubator temperature from 3 to 30 days.

Rabbits can be highly immunized against the herpes virus by repeated subcutaneous injections of normal rabbit serum-virus mixtures prepared from infected brains. The animals become immune to concentrated doses of fresh virus administered subdurally.

The serums from such hyperimmunized animals may exhibit marked virucidal properties and completely neutralize the fresh virus when left in contact with it for as short a time as 3 days at ice box temperature.

Attempts to culture the virus on Noguchi's medium at various reactions at different temperatures, under aerobic and anaerobic conditions have failed.

Cross immunity experiments with the LeFèvre herpes virus and the Levaditi so-called encephalitis virus have been positive, confirming the work of others in establishing the biological identity between these two viruses.

The herpes virus is markedly susceptible to the action of ultra violet rays as generated by the Alpine sun lamp. Exposures of supernatants from concentrated brain emulsions in salt solution for only five minutes destroy the virus. Test animals surviving subdural injections of rayed virus are found to possess no immunity when subsequent injections are given with the fresh virus.

A method of studying experimental encephalitis in the early stages of the disease is described.

Vesicular fluids obtained from cases of herpes zoster when injected subdurally into rabbits have given negative results.

THE NATURE OF BACTERIOPHAGE

TWO PLATES

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In a recent communication [1] we have called attention to the action of ultraviolet light upon the bacteriophage. Exposure, for forty minutes at one foot distance, of a given concentration of bacteriophage active against a strain of B. coli which we designated "B. coli D" because of its diffuse growth in broth, was sufficient to destroy the lytic principle. It was further shown that this principle was protected from the action of the ultraviolet rays when placed in serum instead of broth. The lytic principle used in these experiments has been more fully described.[2]

The experiments to which we refer above have caused us to examine more closely the idea that the bacteriophage is a particulate substance. In the last several years the experimental work which has been reported dealing with this subject has narrowed down to two main schools of thought: d'Herelle [3] has maintained since he published his first work on the bacteriophage that the lytic principle is a living ultramicroscopic virus. The work upon which he bases his opinion is well known and needs no further discussion here. Bordet has been just as insistent in his belief that the lytic principle is of a diastatic nature and is brought about by an interaction between the organism and the tissue cells of the host. Our own work has seemed to favor the Bordet conception and to our minds we must await further proofs before we are able to accept d'Herelle's hypothesis that we are dealing with an ultramicroscopic virus.

D'Herelle [4] has described an experiment which seems to us to be very significant. He states:

> Having given, for example, a culture containing 100,000,000 ultra-microbes per cubic centimeter, a dilution containing a hundred-millionth of a cubic centimeter introduced into a bacillus culture provokes lysis. If one introduces a quantity ten times weaker into ten separate cultures of bacilli, one obtains, on the average, bacteriolysis in but one of the ten cultures. The other nine show no change,

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[1] McKinley, E. B.; Fisher, R., and Holden, M.: Proc. Soc. Exper. Biol. & Med., 1926, 28, p. 408.

[2] McKinley, E. B.: Compt. rend. Soc. de biol., 1925, 93, p. 1052.

[3] The Bacteriophage (Translation), 1922.

[4] Immunity in Natural Infectious Diseases, 1924, p. 253.

giving normal bacillus cultures and the subcultures from them are normal. This experiment is additional proof that the bacteriophage is composed of particles, of which only a single one is liable to effect lysis, but if this particle is lacking, no change, however weak, is observed in the bacterial culture. The lytic principle is then represented by a particle which multiplies; and an invisible particle which multiplies can only be an ultra-microbe.

Bronfenbrenner and Korb [5] have recently offered similar experiments to show that the bacteriophage is particulate and further demonstrate that the concentration of agar determines somewhat the size and number of plaques. By increasing the concentration of agar from 0.3 to 2.5% the number of plaques of lysis was reduced more than one hundred times and the average size of the plaques was decreased to one-tenth of the original. Marshall [6] has also presented recently a comprehensive report based on quantitative studies with the bacteriophage and concludes that the lytic principle is particulate and is in fact a living ultramicroscopic virus.

It seems apparent that such experiments, if true, are sufficient to prove beyond all question of doubt that the bacteriophage is particulate. However, it does not follow that such particles must, and can only be living ultramicrobes. To be sure reproduction is a property of all living matter but are we sure that we are dealing here with reproduction or multiplication in the sense we usually apply those terms to living cells? One must seriously question such an assumption until more definite criteria are available. In 1923 attention [7] was called to the zymogenic granulations of the pancreas and the presence of bacteriophage in filtrates prepared from this organ. Pico,[8] Combiesco,[9] and Bachman and Aquino [10] had previously reported the presence of bacteriophage in commercial preparations of pancreatin and trypsin. We were unable to confirm these results with commercial products.[11] According to Altmann zymogenic granulations may be bioplasts, independent elements, capable of reproduction by division in a cellular medium. If we assume that the bacteriophage is a bioplast do we necessarily have to assume that it is an ultramicroscopic virus? The fact that the bacteriophage parasitizes bacteria would, at first glance, lead us to think so, but are we not justified to the same degree in thinking of zymogen, coming from zymogenic

[5] J. Exper. Med., 1925, 42, p. 483.
[6] J. Infect. Dis., 1925, 37, p. 126.
[7] McKinley, E. B.: J. Lab. & Clin. Med., 1923, 9, p. 185.
[8] Compt. rend. Soc. de biol., 1922, 86, p. 1106.
[9] Ibid., 87, p. 17.
[10] Ibid., 86, p. 1108.
[11] McKinley, E. B.: J. Bact., 1923, 8, p. ??.

granules as living matter since it will digest other types of plant cells? We would not think of calling this substance or the granules from which it originates a virus in the sense we are accustomed to use the term.

In the following experiments we have repeated the experiment of d'Herelle which we have quoted above. The lytic principle we have employed was essentially the same as that used in our experiments with ultra-violet light except that the titer was considerably higher.

Preliminary titrations were made as follows: Agar slants were covered with one drop of a broth culture of a susceptible strain of B. coli, and incubated for two hours. At the end of this period one drop of the bacteriophage dilutions, suspended upon a capillary pipet, was streaked down the center of the young slant culture. After 24 hours in the incubator the tubes were examined for lysis. It will be noted in figure 1, that the lytic principle was markedly active in the 1:10,000 dilution and that several isolated lytic plaques were obtained in the 1: 100,000 dilution. The higher dilutions were negative.

It must be remembered, however, that in these tests we have only examined one drop—we have not examined every drop—and if the bacteriophage is particulate and if it is unevenly distributed throughout the substrate we might expect either its absence or its presence in single drop quantities when certain dilutions are reached. That this is true we will show later. Assuming then that the lytic principle we were using was active in dilutions of 1: 100,000 as tested by the drop method, we devised the following experiment which is similar to that of d'Herelle's.

Beginning with 1 cc. of the concentrated bacteriophage we prepared a dilution of 1: 10 by taking .1 cc. of the concentrated material and adding .9 cc. of broth. From the 1: 10 dilution a 1: 100 dilution was prepared in a similar way. From the 1: 100 dilution we prepared a 1: 1,000 and so on from each dilution until we had reached a dilution of 1 10,000,000. Our experiment then was to test for the presence of bacteriophage in every 1 cc. quantity of these dilutions and so account for every particle which was present in the first 1cc. of concentrated material. If the lytic principle were absent in any part of any dilution we could conclude that we were not dealing with a substance in true solution.

It will be noted in table 1 that particles of bacteriophage were absent in four different .1 cc. quantities when tested on young agar plate cultures of the sensitive organism. This was in a dilution of 1: 10,000,000. There were seven plaques in one .1 cc. quantity, 3 plaques in another, 4 in another and one each in two other quantities. Due to the difference in the capacity of our pipets these quantities really represent $\frac{1}{9}$ cc. The tenth plate was tested with .1 cc. of broth used to wash out the dilution tube and the pipet. The total number of bacteriophage particles present in 1 cc. of a 1: 10,000,000 dilution was found to be 16. This number

should equal the average number of particles present in the next lower dilution since .1 cc. of this dilution was carried over to make up the 1 : 10,000,000 dilution. This is not found to be the case since the total number of plaques obtained from .9 cc. of 1 : 1,000,000 dilution was 68 and the average (counting only the first eight quantities since the last two were wash quantities) was 8.5 plaques. However, it is to be noted that the number of units present in the different quantities varies from 3 to 12 showing a very uneven distribution. We know that we took at least 16 units over into the 1 : 10,000,000 dilution since we recover that

TABLE 1

Number of Lytic Plaques on Plates of Every .1 cc. of Each Progressive Dilution (1 cc.) of Bacteriophage

Dilutions of Bacteriophage	Plates									
	1	2	3	4	5	6	7	8	9	10
Concentrated	+	+	+	+	+	+	+	+	+	+ w
1:10	+	+	+	+	+	+	+	+	+	+ w
1:100	+	+	+	+	+	+	+	+	+	+ w
1:1000	+	+	+	+	+	+	+	+	+	+ w
1:10,000	20*	479	467	356	475	367	357	274	50 w	90 w
1:100,000	55	60	70	67	58	60	81	13	20 w	24 w
1:1,000,000	12	11	7	8	13	4	9	3	0 w	1 w
1:10,000,000	0	7	0	0	0	3	4	1	1	0 w
Repetition with Second 1 cc. of Lytic Principle										
Concentrated	+	+	+	+	+	+	+	+	+	+ w
1:10	+	+	+	+	+	+	+	+	+	+ w
1:100	+	+	+	+	+	+	+	+	+	+ w
1:1000	+	+	+	+	+	+	+	+	+	562 w
1:10,000	677	631	597	652	671	493	587	593	82 w	111 w
1:100,000	93	74	91	90	76	95	86	52	7 w	11 w
1:1,000,000	12	13	6	11	4	10	4	8	0 w	2 w
1:10,000,000	2	1	0	1	0	1	1	0	0 w	2 w

+, Large lytic areas, no plaques. Isolated colonies of B. coli "D."
w, Washings from dilution tube and pipette.
* Mixture of plaques and large lytic areas. Accurate count not possible.

many plaques. The total number of plaques (68) in the 1 : 1,000,000 dilution is not far removed from the average number of plaques obtained from the 1 : 100,000 dilution which was 50.8.

In a second experiment (table 1) using a second 1 cc. quantity of the concentrated lytic principle and following the same procedure, we obtained even better results. One notices that the lytic particles were absent in three quantites of the dilution 1 : 10,000,000. Figure 6 shows two of these plates in which there are no lytic plaques. Figure 5 also shows series H, dilution 6, in which there is a single plaque to be found a half centimeter below the first comma in the label. The total number of plaques in this dilution was 8 and the average number of plaques in the next lower dilution was 7. The total number of plaques in the

1 : 1,000,000 dilution was 70 while the average number of plaques in the next lower dilution 1 : 100,000 was 67.5. These figures are fairly close though as we reach the more concentrated dilutions the error becomes greater. For example the total number of plaques in the 1 : 100,000 dilution was 657 by our count while the average number of plaques in the 1 : 10,000 dilution was 509.4 showing a much larger error. In neither the first nor the second experiment were we able to even approximate the lytic activity in the concentrated material or in the 1 : 10, 1 : 100 or 1 : 1,000 dilutions (figs. 2, 3).

In the first experiment we obtained 16 plaques in our 1 : 10,000,000 dilution while this dilution in the second experiment gave only 8. These two quantities of 1 cc. each were removed from a 500 cc. flask containing our stock bacteriophage at different times and serves to illustrate in another way the uneven distribution of bacteriophage particles.

We were also interested in knowing what each of these plaques represented in terms of particles of bacteriophage and so several of the plaques were punched out of the agar plate with small sterile capillary pipets. The small agar plug was blown out of the capillary into a small test tube which contained three or four small BB shot and .5 cc. of broth. The open end of the tube was then sealed and the agar plug supporting the plaque was mechanically broken up until the liquid was turbid. This material was then spread over the surface of young agar plate cultures of the sensitive organism and incubated for 24 hours. The following day it was found that these plates gave growths of isolated bacterial colonies similar to the concentrated material. Only on one plate were we able to count any plaques and there were three, while the rest of the plate showed marked evidence of lytic activity such as we obtained with the most concentrated principle. Arnold [12] has shown that the lytic substance diffuses through the agar so we are not surprised at these results. Plugs of agar and culture but without bacteriophage plaques used as controls gave normal culture plates.

Hadley [13] has pointed out that the sensitive strains present in cultures are apparently stimulated to greater activity and the colonies show marked evidence of proliferation when in contact with the bacteriophage. We have noticed this phenomenon in our series A, B and C plates where the concentration of the bacteriophage was highest. The same phenomenon can be demonstrated in respiration experiments where an alkali trap is used. The cultures of B. coli for example which are in contact with

[12] J. Lab. & Clin. Med., 1923, 8, p. 720.
[13] J. Infect. Dis., 1925, 37, p. 35.

the bacteriophage produce much more CO_2 than the cultures without bacteriophage. Macroscopically there is a much greater growth after 24 hours' incubation in the broth tubes containing both the organisms and bacteriophage than in those tubes containing only organisms.

The appearance of the resistant strain in the center of lytic plaques after several days' ageing is well known. Brown and Basaca [14] have recently reported this same phenomenon in connection with pseudo-bacteriophage of B. anthracis and believe the center of the plaque in the early stages is composed of anthrax spores. These various observations may in time lead us to attach more importance to the role played by the resistant strains in our cultures in the production of bacteriophage. While the lytic principle apparently increases at the expense of the sensitive strains its origin may be from the resistant strain which has been modified by environmental conditions such as passing through the intestinal tract. If some such explanation were possible it would explain such instances as B. coli Lisbonne which is able to produce a lytic substance for B. Shiga dysenteriae.[15] Brown and Basaca describe the center of the anthrax plaques as being granular and Fontes [16] has recently reported in the life cycle of bacteria a granular stage which precedes the phase of growth. It is to be hoped that further experiments will correlate some of these phenomena and provide an explanation for what seems to be in many instances a paradoxical activity.

CONCLUSIONS

We concur with d'Herelle that the bacteriophage is a particulate substance. Being particulate does not in and of itself imply its living nature though its power of multiplication and adaptation are strong arguments for it. We question the theory of its being an ultramicroscopic virus in that adequate proof is lacking for this conception. However, it is possible to conceive of an ultramicroscopic particulate substance, diastatic in its action, granular in form and inanimate, which may be derived from the bacterial cell during a stage in its life cycle as a result of the influence of tissue cells of the host have had upon it. Bordet has called this "hereditary vitiation" and it may well be. The susceptibility of the bacteriophage to the action of ultraviolet rays is suggestive of its living nature though it is well known that enzymes are also destroyed by them and such a criterion would not serve in proving this point.

[14] Proc. Exper. Biol. & Med., 1926, 22, p. 625.
[15] McKinley. E. B.: Compt. rend. Soc. de biol., 1925, 93, p. 1050.
[16] Mem. Inst. Oswaldo Cruz, 1925, 18, p. 163, 197.

PLATE 1

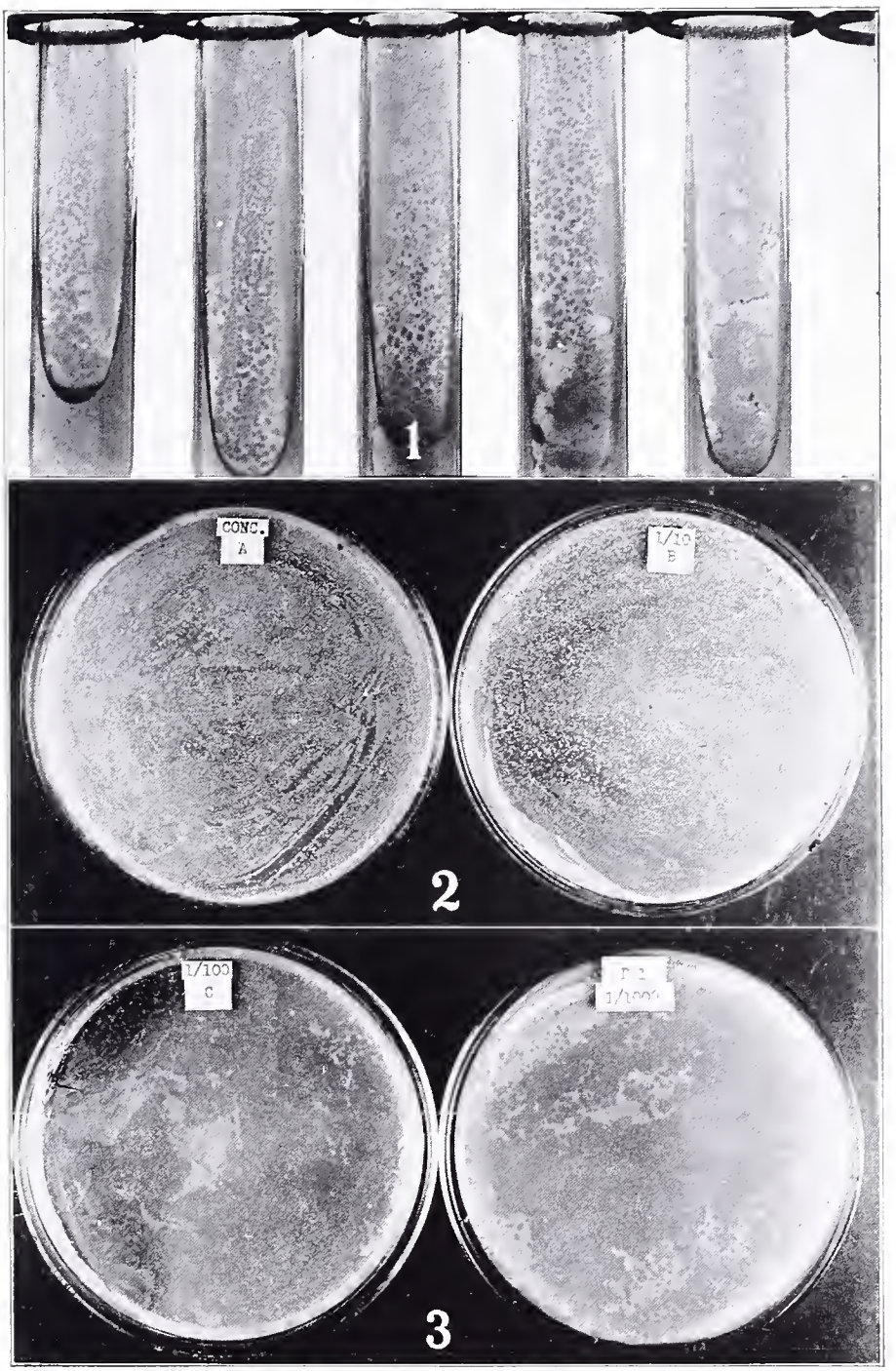
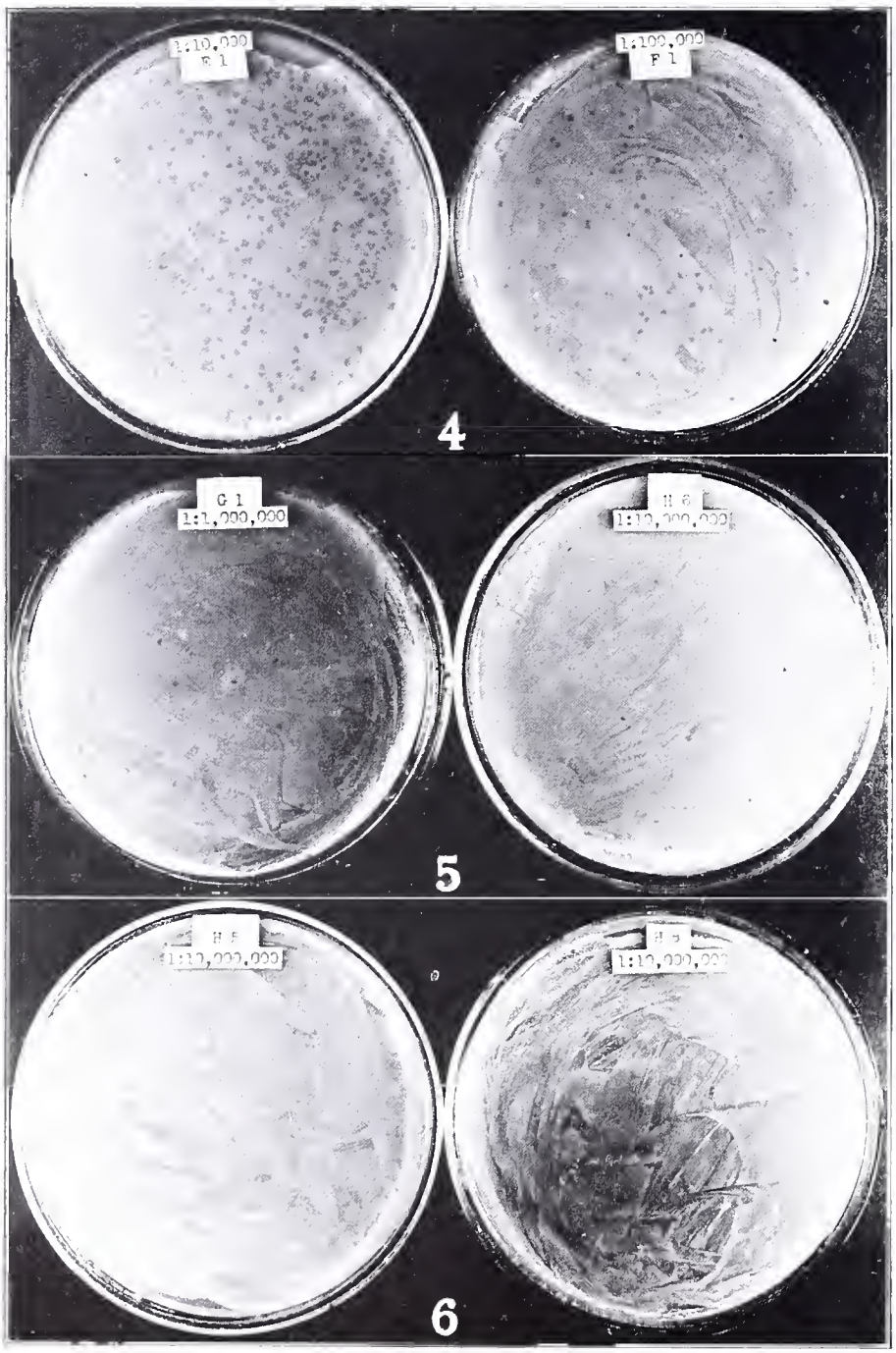


Fig. 1.—Preliminary titration with single drop from dilutions.

Figs. 2 and 3.—Plates with progressive dilutions of second 1 cc. of lytic principle (table 1).

PLATE 2



Figs. 4-6.—Plates with higher dilutions, also from repetition with second 1 cc. of lytic principle (table 1).

A CULTURAL STUDY OF THE ACETONE BUTYL ALCOHOL ORGANISM

ONE PLATE

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The anaerobic butyric acid bacteria divide naturally into two main groups: those forms which produce chiefly butyric and acetic acids, and those forms which produce chiefly neutral end products, butyl alcohol and acetone. This simple method of dividing the butyric bacteria has received little consideration. In many painstaking studies the investigators have not definitely identified their cultures. The resulting confusion regarding the names and classification of these bacteria, and the improved methods for the culture of anaerobic bacteria, made the study of a large number of cultures of the butyric acid bacteria seem worth while. The present paper discusses only the representatives of the second group, which produce the neutral substances, acetone and butyl alcohol. The characteristics of the first group, acid-forming butyric bacteria, will be reported later.

The property of certain butyric bacteria to form butyl alcohol from carbon compounds has long been known. Pasteur[1] considered butyl alcohol as an ordinary product of butyric fermentation. "Il croit pouvoir affirmer que l'alcool butylique est un produit ordinaire de la fermentation butyrique."

Fitz[2] described a butyl alcohol forming organism, Bacillus butylicus, which was granulose-positive, motile, formed heat-resistant spores, and attacked glycerol, mannitol, and sucrose, but not calcium lactate, lactose, or starch. The products were chiefly carbon dioxide, hydrogen, normal butyl alcohol, normal butyric acid and small amounts of ethyl alcohol.

Twenty-five years after Pasteur, Gruber[3] described three strains of butyl alcohol-forming bacteria under the name Clostridium butyricum (B. amylobacter). Two of these, types 1 and 2, were anaerobic and showed a distinct granulose reaction. His description is too meager, however, to identify the types he mentioned. Botkin[4] isolated and described an anaerobic granulose-positive organism producing butyl and ethyl alcohol, again with a description hardly sufficient for

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[1] Bull. Soc. Chim. de Paris, Seance du 9 mai, 1862, p. 52.
[2] Ber. d. deutsch. Chem. Gesellsch., 1878, 11, p. 42; 1882, 15, p. 867.
[3] Centralbl. f. Bakteriol., I, O., 1887, 1, p. 367.
[4] Ztschr. f. Hyg., 1892, 11, p. 421.

identification. In many respects his Bacillus butyricus is similar to the acetone-butyl organism described in the present paper.

Grimbert[5] then described a motile, anaerobic, spore-forming organism which did not stain blue with iodine at any period in its development. This organism, named Bacillus orthobutylicus, fermented glycerol, mannitol, starch, lactose, and other sugars with the production of carbon dioxide and hydrogen, normal butyl alcohol, butyric acid, and acetic acid. The organism did not ferment calcium lactate. Grimbert studied the effect of duration of fermentation, reaction, concentration of carbon compounds, and age of the seed on the proportion of the fermentation products. He found a decrease in butyl alcohol when the fermenting culture was neutralized with $CaCO_3$.

A report by Beijerinck,[6] concerning the butyl alcohol-forming bacteria, deserves special emphasis. He describes four species which he named Granulobacter butylicum, Granulobacter saccharobutyricum, Granulobacter lactobutyricum, and Granulobacter polymyxa. The first may be considered the type. He created the term "Granulobacter" to replace the genus name Clostridium and applied it to those organisms which, under anaerobic conditions, showed a blue color with iodine, and also exhibited swelling of the cell at sporulation.

With the exception of Granulobacter lactobutyricum, all of these organisms produced normal butyl alcohol. Beijerinck suggested the identity of his Granulobacter butylicum with Gruber's Bacillus amylobacter 1 and Granulobacter saccharobutyricum with Fitz's Bacillus butylicus. He found that these bacteria would entirely reduce litmus and were decidedly anaerobic. The addition of a strong aerobic microorganism, Saccharomyces mycoderma, favored their development.

Duclaux[6a] isolated from potato a granulose-positive butylic organism which changed 25 to 30% of the starch destroyed into butyl alcohol. Other products were hydrogen and carbon dioxide, acetic and butyric acids, and ethyl alcohol. Assuming that one organism could not produce two alcohols, he believed that he had two associated organisms, Amylobacter butylicus and Amylobacter ethylicus, which could not be separated. His culture attacked sucrose, lactose, maltose, starches from various sources, mannitol, glycerol, and calcium lactate. He also noted that $CaCO_3$ decreased the yield of butyl alcohol.

Grassberger and Schattenfroh[7] produced butyl alcohol with a motile butyric acid organism which they had isolated from soil; however, later experiments with the same culture failed to show butyl alcohol.

Within the limited space of this report it is possible to give a summary of only the early investigations concerning the butyl alcohol-forming bacteria. Attention is, however, called to the later contributions of Buchner,[8] Buchner and Meisenheimer,[9] Emmerling,[10] Winogradsky,[11] Pringsheim,[12] Bredemann,[13] Henneberg,[14] Kirow[15] and others. In general the organisms studied by these workers proved identical with or closely related to Bacillus butylicus (Fitz[2]). The recent con-

[5] Ann. de l'Inst. Pasteur, 1893, 7, p. 353.
[6] Verhandel. d. Koninkl. Akad. v. Wettenschappen (Sect. 2), 1893, 1, p. 1.
[6a] Ann. de l'Inst. Pasteur, 1895, 9, p. 811.
[7] Arch. f. Hyg., 1902, 42, p. 219.
[8] Ztschr. f. physiol. Chem., 1885, 9, p. 395.
[9] Ber. d. deutsch. Chem. Gesellsch., 1908, 41, p. 1410.
[10] Ibid., 1897, 30, p. 451.
[11] Centralbl. f. Bakteriol., 2, O., 1902, 9, pp. 3, 107.
[12] Ibid., 1906, 15, p. 300; 16, p. 795.
[13] Ibid., 1909, 23, p. 385.
[14] Gärungsbakt. Prakt. Betrieb. u. Pilzkunde, 1909, p. 568.
[15] Centralbl. f. Bakteriol., 2, O., 1911, 31, p. 534.

tributions of Speakman,[16] Thaysen,[16a] Fowler,[17] Neuberg,[18] Robinson,[18a] and their associates have done much to explain the biochemistry of the acetone-butyl-alcohol fermentation.

Little attention has been given to the systematic study of the butyl alcohol organisms. From the excellent papers on the anaerobic bacteria which have appeared recently many useful methods have been adopted for this study of eleven cultures from widely separated sources. Because of the difficulty in culturing these organisms, it is not surprising that the individual reactions of the acetone butyl alcohol organism are not well known.

Seven strains of the acetone butyl alcohol organism were obtained from the Commercial Solvents Corporation of Terre Haute, Indiana, and 4 strains from the Department of Agricultural Bacteriology at the University of Wisconsin, as follows.

Strain	Laboratory Number	Source	Date of Isolation
1	50	Corn	1915
2	55	Molasses	1920
3	60	Corn mash	1923
4	65	Melle, France, source unknown	1918
5	70	Unknown	1922
6	100	Garden soil	1923
7	105	Barley	1919
8	150	Mud of Lake Mendota	1920
9	180	Barley	1923
10	200	Field soil	1923
11	300	Potato	1923

The purity of the cultures has been a matter of concern throughout this investigation. Aerobic contamination has been easy to eliminate, in the first place by selective heating for the nonsporeformers and by selective cultural conditions or presence of dyes for the sporeformers. There has been no difficulty with aerobic contamination, as aerobic control plates have frequently proved. Assurance of anaerobic purity is not so simple. Only one strain of the butyl organism (8) has been cultured from a single spore. The other ten strains have been purified by repeated picking of colonies from deep agar plates or from dilution tubes of agar. They have shown no sign of impurity and have differed in no way from strain 8, our culture from a single spore. It would seem, then, that careful and repeated picking of deep colonies of anaerobes is an entirely satisfactory method of isolation, as Hall[19] and Brown[20] have reported it to be.

Although the terms Bacillus granulobacter pectinovorum, Bacillus amylobacter, Clostridium butyricum, Bacillus butyricus, and many other names have been used for the acetone-butyl alcohol bacteria we prefer to use the common name, butyl organism.

The plan of study has been to apply the tests of the descriptive chart of the Society of American Bacteriologists, other tests suitable for anaerobic classification, and any methods that might be helpful in distinguishing one type from another.

[16] J. Biol. Chem., 1923, 58, p. 395.
[16a] J. Inst. Brewing, 1921, 27, p. 529.
[17] J. Ind. Inst. Sc., 1925, 8A, p. 71. Subramangan, V.: Some Studies in Biochemistry, 1924. Gokhale, A. G.: Some Studies in Biochemistry, 1924.
[18] Biochem. Ztschr., 1921, 117, p. 269.
[18a] J. Biol. Chem., 1922, 53, p. 125.
[19] J. Infect. Dis., 1920, 27, p. 576.
[20] J. Bact., 1925, 10, p. 513.

Standard medium formulas in the Manual of Methods for Pure Culture Study of Bacteria and those recommended by others for anaerobic culture have been carefully followed. Wherever possible, reference is made to the paper in which the medium was first described. Modifications which have been made are indicated.

Synthetic medium A, a modification of Speakman's [16] formula, has been used unless otherwise stated.

Formula	Gm.	Formula	Gm.
K_2HPO_4	0.50	$MnSO_4$	0.01
KH_2PO_4	0.50	Peptone	5.00
$MgSO_4$	0.20	Water	1000.00
NaCl	0.01	Carbohydrate	3%
		Reaction	Ph 7.0

The methods of culture have been made as simple as possible for obligate anaerobic bacteria.

Generally a deep medium, seeded at the bottom is satisfactory by reason of its depth and because of the vigorous gassing of the organisms. For surface growth the tubes were incubated in anaerobic jars in which anaerobiosis was obtained by the potato respiration method (Carroll and Hastings [21]), or by evacuation. In some of the fermentation work cultures were also grown under vaseline seals.

Variation in activity of the butyric bacteria depending upon the source of cultures has been noted repeatedly. Cultures from soil have, for example, shown the ability to fix atmospheric nitrogen while the same organisms cultured for a long time on artificial mediums lost this property. For each of our tests one-half of the cultures were inoculated with the third or fourth subculture started from spores in corn mash and one-half with similar subcultures from spores in soil. The cultures from soil usually proved the most vigorous. The results are the average of duplicate cultures from different batches of medium; often these tests were repeated 3 or 4 times.

GENERAL CHARACTERISTICS

One of the most distinctive features of the group to which the butyl organism belongs is the granulose reaction. Young vegetative cells (plate 1, figs. 1, 2) stain yellow with iodine. As sporulation approaches and the cells take on clostridial form, they store granulose and stain blue or violet with iodine. As the spores mature, the granulose reaction is lost, indicating that the reserve material has been utilized in sporulation.

Young vegetative cells are actively motile with 9-18 peritrichous flagella (plate 1, fig. 4). They are gram-positive. After 18 to 24 hours shrivelled gram-negative cells increase in numbers. These are probably inactive or dead cells, for differential staining with Congo red has shown a parallel increase in numbers of dead cells. Cultures of the

[21] J. Lab. & Clin. Med., 1925, 11, p. 264.

butyl organism may be slimy, but capsules cannot be demonstrated by any positive method. Under parallel conditions Cl. welchii cells have shown capsules.

The dimensions of the cells of a given bacterial species are affected more or less by the environment. Therefore one cannot specify the dimensions of an organism without stating the age, medium, staining method, and other conditions under which the observations were made. A study of the developmental changes of strain 7 in corn mash has been made at 3 to 6 hours intervals from the time of inoculation to the 75th hour of fermentation. For purposes of measuring, the negative nigrosin mounts made without heat are most satisfactory. The averages of many measurements show the following trend of change in the size of cells in a corn mash fermentation:

Time After Inoculation	Cell Types	Dimensions (Microns)
3 hours	Young vegetative rods	4.7 x 0.72
	Granulose negative	
27 hours	Vegetative rods	3.4 x 0.66
	Clostridia. Granulose-positive	4.7 x 1.6
75 hours	Vegetative rods	2.6 x 0.6
	Clostridia	5.5 x 1.3
	Free spores	2.4 x 1.2

With the Dorner [22] spore stain, the development of spores is easily followed (plate 1, fig. 5). Young clostridia are completely decolorized with nigrosin. By the 6-9th hour certain irregular granules, one or more in a cell, retain the stain. Gram mounts of the same age show the granules to be gram-positive, and iodine proves them to be reserve granulose. Gradually they fuse and assume the regular outline and dimensions of spores—large oval bodies somewhat displaced from the center of their sporangia. After the 30-33rd hour free spores are found, their numbers increasing as the clostridia degenerate (plate 1, fig. 3). Comparatively few of the organisms in corn mash reach the spore state.

The literature contains repeated references (Pringsheim [12]) to two or more spores in clostridia of the butyric acid anaerobes. Some investigators have pictured occasional cells with spores at both extremities and Grimbert [5] has referred to 2 or 3 spores in a cell as a regular characteristic of his organism. Omeliansky,[23] discussing Cl. pasteurianum, suggests that a cell with two spores is in reality two cells. The granules of the early stages of spore formation of the butyl organism might indicate multiple spores, but when sporulation is complete the granules have fused to a single spore in a cell.

[22] Le Lait, 1926, 6, p. 8.
[23] Arch. d. Sc. Biol., 1916, 20, p. 24.

The rate of growth in corn mash fermentations has been followed by direct microscopic counts by the Fries [24] method. The corn mash inoculum of 1 : 45 gave an initial count of 33 millions per cc., which probably explains the lack of a lag phase. The bacilli develop rapidly, reaching a peak of over two billion cells per cc. At the same time the acidity rises to a peak and then drops sharply as the solvent production sets in. The growth curve, on the other hand, falls gradually as the active fermentation ceases. The relation of the growth curve and acidity curve is illustrated in text figure 1.

Colony structure is of limited value in description of species because it is influenced by so many physical factors. Brown [20] concludes that

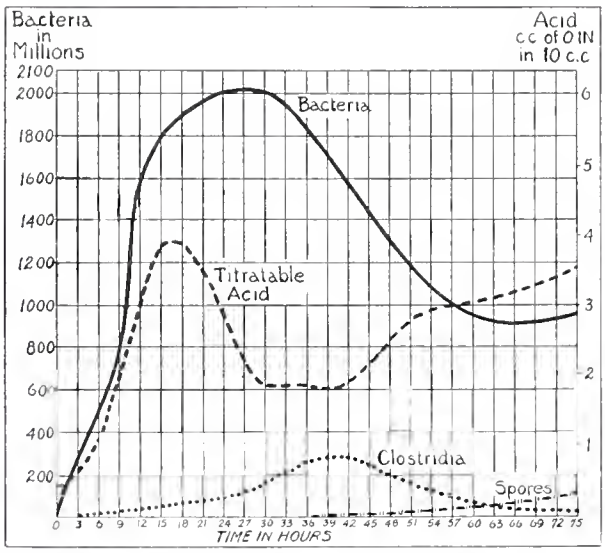


Fig. 1.—Relations of rate of reproduction and formation of titratable acids in corn mash.

there are only two types of deep colonies of anaerobes—round, compact, biconvex; and diffuse, rhizoid, woolly. Minor differences between or within species are insignificant. Deep colonies of the butyl organism are of the compact type. Surface colonies, less influenced by their environment, are compact, raised, and fairly regular. Colonies between the agar and glass of a Petri dish are round and spreading with a border of wavy markings, which may be characteristic.

Oxygen Relations.—The oxygen relations of the butyl organism are somewhat paradoxical. It is certainly less sensitive to air than are certain of the pathogenic anaerobic spore-formers, for it can be cultured

[24] Centralbl. f. Bakteriol., I, O., 1921, 86, p. 90.

in open tubes of fresh carbohydrate media. Germination of spores in air is not possible as in the case of ordinary facultative organisms. And yet after a culture is well started in corn mash, oxygen may be bubbled through it intermittently without seriously disturbing its fermentation. Likewise, colonies developed in an anaerobic jar may on removal to air continue to grow by a piling up of the bottom layers of growth. Large inocula are advantageous because they provide initial points of growth and gassing. From this it may be inferred that, given a strictly anaerobic beginning, each culture of the butyl organism maintains its own anaerobic conditions. On the other hand, the requirements of the organism may vary with its stage of development or with the medium offered.

Reaction of Medium.—Normally the acid production and acid utilization of the butyl organism are delicately balanced, and acid does not accumulate. If there are present with it certain high acid-producing organisms, the normal sequence of changes is blocked; the culture becomes obviously sick; and the yield of solvents is reduced. The extreme P_H values for growth have not been determined. For most of the laboratory mediums a P_H of 6.0-7.0 is satisfactory. Growth in corn mash occurs between P_H 4.7 and 8.0, although solvent yields at both extremes are decreased. Calcium carbonate has a marked effect on the balance of products: the yield of alcohol and acetone is depressed and the calcium salts of the acids accumulate in the medium.

Temperature.—For ordinary laboratory cultures as well as for large scale fermentations of corn mash, 37 C is entirely satisfactory. Apparently normal fermentations may be run a few degrees above or below this temperature. Gelatin cultures were successfully grown at 20 C and probably could have been grown at a still lower temperature. The marked decrease in rate of growth at lower temperatures may prove to be of advantage in studies of nitrogen fixation. For a few degrees rise in temperature above 37 C there is no serious effect on the organism. The balance of products and the appearance of culture may be somewhat changed at 40-47 C, but on being returned to 37 C the cultures revert to normal. At 65 C, a temperature too high for growth, there is slight enzymatic action on the corn mash. The organism in its spore state in corn mash resisted 65 C for 40 days and appeared normal on return to 37 C.

Reduction of Dyes.—The butyl organism has pronounced reducing power either by enzymatic action or by liberation of active hydrogen.

The rate of methylene blue reduction runs parallel to the increase and decrease in numbers and activity of the organisms. At the height of fermentation complete reduction is accomplished in a few minutes while at the beginning and end several hours may be necessary. Active cultures reduce methyl red almost instantaneously and brom cresol purple or brom phenol blue within a few minutes. Litmus in milk is completely reduced within 24 hours, but is reddened again as the cultures stand in contact with air.

Thermal Death Point of Spores.—Selective heating has been commonly practiced in the isolation of the butyric acid bacteria. As noted above the spores of the butyl organism will stand 65 C for many days; they easily survive 80 C for 10 to 30 minutes. Their resistance at 100 C has been studied by Hastings, Fred, and Carroll.[25] Under their specified conditions the thermal death point in 3% corn mash is approximately 4 minutes at 100 C.

Proteolysis.—Chemical tests have been used for the detection and quantitative measure of the products of protein decomposition, such as proteoses, amino acids, ammonia, indol, hydrogen sulfide, and skatol. The value of such tests when properly made can hardly be questioned, but they require much time and skill. A quantitative study of the proteolytic action of the butyl alcohol organism on animal and vegetable proteins has been carried out by Peterson, Fred, Domogalla,[26] and by Fulton, Peterson, and Fred,[27] whose results showed an extensive proteolysis of all of these proteins. The less complicated qualitative tests are not so definite but are widely used. A foul odor, as the criterion of proteolysis, is largely a matter of personal interpretation. Liquefaction is more suitable because it can be described in definite terms. Liquefaction of gelatin and digestion of coagulated albumin, serum, brain or meat media are especially useful tests of proteolysis by anaerobic bacteria.

Gelatin: Gelatin is a simple and incomplete protein and hence the ability of an organism to hydrolyze gelatin is no indication of its ability to hydrolyze other proteins.

In the study of the butyl organism the following have been used: peptone beef-extract gelatin (plain gelatin), 0.25% glucose beef-extract gelatin, Bredemann's gelatin,* and beef extract gelatin adjusted to + 1 Fuller scale. With the exception of + 1 gelatin, all were adjusted to P_H 7.0. The cultures were incubated at 20 and 37 C.

[25] Centralbl. f. Bakteriol., 2, O., 1926, 67, p. 162.
[26] J. Am. Chem. Soc., 1924, 46, p. 2086.
[27] Centralbl. f. Bakteriol., 2, O., 1926, 67, p. 1.
* Peptone (Witte), 12.0 Gm.; Liebig's beef extract, 8.0 Gm.; sodium chloride, 2.0 Gm.; glucose, 10.0 Gm.; gelatin, 200.00 Gm.; water, 1,000 c c.

With a 1:60 inoculum at 37 C all strains liquefied plain and glucose gelatin within 24 hours, and the more concentrated Bredemann gelatin within 24 to 48 hours. When the inoculation is by needle, growth is slow and uncertain, and liquefaction is very much delayed. In one comparative experiment liquefaction of a + 1 gelatin was delayed for 22 to 25 days.

At 20 C liquefaction was slower, but as complete as at the higher temperature. Fully 48 hours were needed for liquefaction of plain and glucose gelatin and 3 or 4 days for liquefaction of Bredemann's gelatin.

Hall [28] and others report that the presence of fermentable sugars may retard or prevent the liquefaction of gelatin. Hall used 0.2% glucose to insure good growth of his anaerobes and found no unfavorable effects. Our results indicate that there is no difference in liquefaction of plain and of 0.25% glucose gelatin. The Bredemann gelatin, containing 1% glucose and 20% gelatin, did not liquefy at 20 C for 3 days, while plain and 0.25% glucose gelatin were liquefied in 48 hours.

Growth of these anaerobic bacteria in plain gelatin without glucose is fair with only a small amount of gas. In glucose gelatin and in Bredemann's concentrated gelatin there is excellent growth, vigorous gassing for two or three days, and a heavy dark precipitate. After 3 or 4 days the medium becomes a light amber color and as clear as broth. Probably acid production is responsible for clearing, but acidity cannot be responsible for liquefaction, because the rapid liquefaction within 24 to 48 hours occurs before very high acidity is reached, and also because related cultures which never liquefied also produced acid during the long period of incubation—42 days.

In plain gelatin at 37 C the following results are typical:

		Cc. N/10 cc. of culture:
Liquefaction in 48 hours, butyl organisms:	7	2.6
	1	3.4
No liquefaction in 6 weeks:	Cl. pasteurianum (Winogradsky)	5.4
	B. amylobacter (Dorner)	5.6

Long incubation at 37 C was not responsible because the uninoculated controls under the same conditions were able to solidify.

Additional support for the above results will be seen in the qualitative chemical tests for gelatin. Picric acid, 10% tannic acid, trichloracetic acid, alcohol, and zinc sulfate in various concentrations were tried. Trichloracetic acid as used by Hiller and Van Slyke [29] gave the clearest distinctions. Equal parts of trichloracetic acid, 25% solution, and of culture were mixed. If gelatin is present, a heavy white precipitate appears; if not, there is no precipitate. All strains of the butyl organism in plain gelatin, glucose gelatin, + 1 gelatin, Bredemann's gelatin,

[28] J. Infect. Dis., 1922, 30, p. 445.
[29] J. Biol. Chem., 1922, 53, p. 253.

and the synthetic A medium with 2% gelatin substituted for peptone, gave no precipitates.

Coagulated Egg Albumin: The liquefaction of coagulated proteins has been emphasized by Kahn [30] and Hall [28] in their classifications of anaerobic bacteria.

Coagulated egg albumin is especially useful in this connection, for cubes may be cut and sterilized in any favorable medium. For the butyl bacteria corn mash, synthetic medium A, nutrient broth, and broth minus peptone were used. After 7 weeks' incubation the pieces of egg were examined. The controls were unchanged. The butyl culture in corn mash and synthetic medium A had caused the egg to soften and turn brown. No foul odor was noted; however, the sourish solvent odor of the fermented mediums may have masked the putrefactive odor. In the broth mediums there had been little or no growth; the egg appeared unchanged.

Coagulated Beef Serum: Coagulated beef serum, or Loeffler's blood serum, is a second coagulated protein which should be included in a study of proteolysis. Both deep tube and streak cultures of 5 representative strains of the butyl organism were studied. Observations were made during 15 days incubation in anaerobic jars, and later after 2 weeks incubation in air.

In the anaerobic jars the growth of all cultures was excellent on the surface of the slants and in the liquid of the deep tubes, but there was no sign of darkening or disintegration. The cultures seemed inactive after the 5th day. However, observations were made over a period of a month to be certain that a slow enzyme action might not be overlooked. There were no changes in color or texture of the coagulated serum during that time.

Growth in Milk.—Milk is a very favorable medium for the growth of the butyl organisms. The addition of litmus makes it more favorable, as it does for other anaerobes (Novy [31]). The appearance of milk cultures varies considerably, depending upon the size of the inoculum, as shown in figure 2.

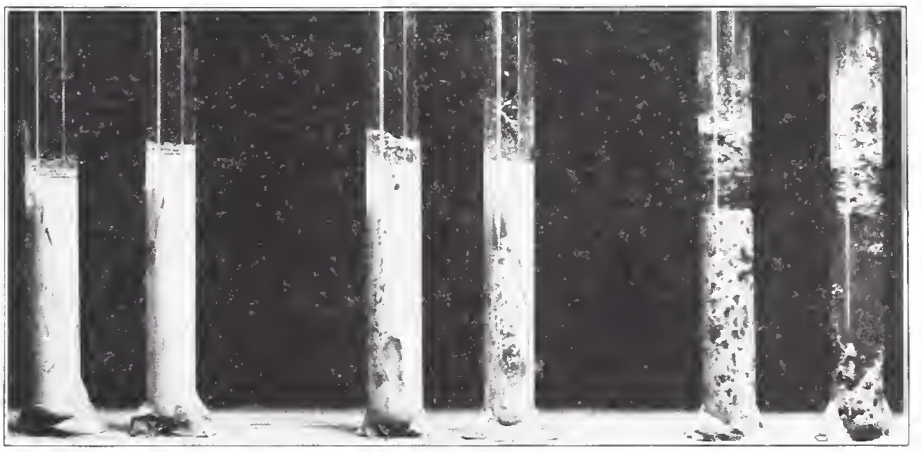
A small inoculum of 2 drops to 20 c c. (1: 180) gave slow curdling and regular gas channeling from bottom to top (fig. 2, group 1). The curd was not badly shrunken and did not appear to change on long incubation. An inoculum of 0.5 c c. to 20 c c. (1: 40) caused more rapid growth and more extrusion of whey (group 2). A large inoculum of 1 c c. to 20 c c. (1: 20) brought about a very rapid fermentation with curd torn by gas and shrunken to a small floating mass (group 3).

Such a stormy fermentation of milk has previously been considered characteristic of Cl. welchii and has been used as a diagnostic test for it

[30] J. M. Research, 1922, 43, p. 155.
[31] J. Infect. Dis., 1925, 36, p. 343.

(Jordan [32]). The distinction should be made between motile and nonmotile anaerobes giving stormy fermentation. Henry [33] has clearly recognized that anaerobes of the amylobacter group may give stormy fermentation but they are distinguished from Cl. welchii by their motility. As a diagnostic test for Cl. welchii the stormy fermentation must be produced by a nonmotile anaerobe.

The amount of whey depends entirely on the shrinkage of the curd. As the cultures grew older, after 7 to 10 days, the whey changed from a turbid to a clear liquid, and a yellow pigment developed. The growth in litmus milk is somewhat more rapid than in plain milk, and as a result there is more extrusion of whey. It is a common thing to find litmus



1 2 3

Fig. 2.—Relation between size of inoculum and kind of curd produced in milk. Ratios of inoculum to amount of milk in tubes 1 (1: 180), 2 (1: 40), and 3 (1: 20).

tubes with small inocula curdled within 24 hours, while parallel tubes of plain milk are not. If observed in time, it can be seen that reduction and curdling proceed from bottom to top. Ordinarily milk is completely curdled and litmus reduced in 24 hours. On standing in contact with air, oxygen gradually works back and the red zone moves down from the top. Tubes kept in an anaerobic jar are completely reduced and do not redden until removed.

Young milk cultures have a pleasant solvent odor and a taste both sweet and bitter. A culture 4 months old had a sourish old cheese odor and a sour and bitter taste. The titratable acidity shows a surprising rise in old cultures.

[32] General Bacteriology, 1925, p. 394.
[33] J. Path. & Bact., 1917, 21, p. 344.

	Strains	C c. N/10 Acid in 10 C c. of Culture
Cultures 4 days old	1	5.8
	3	6.4
	7	6.6
	8	6.1
Cultures 4 months old	1	15.2
	5	16.1
	7	16.5

To all appearances, there is no digestion of the curd, even in tubes incubated for 4 months. But the butyl organisms are known to be proteolytic. To settle the point of casein hydrolysis all strains were plated on milk agar. Two mediums have been used: one of milk and beef peptone glucose agar, equal parts, and the other of milk and 3% agar, equal parts. The plates were incubated in an anaerobic jar and examined after 48 hours. In the medium containing both peptone and casein there was no sign of clearing. In fact, the lines of growth were surrounded by an opaque zone of casein precipitated by acid. Evidently in the presence of other sources of nitrogen, peptone, and beef-extract, the casein of milk was not attacked. In the plain milk and agar medium, however, there were clear zones around all areas of growth. Such a clear zone on a milk agar plate may mean either true hydrolysis of casein, or the formation of the mono-acid salt of casein which is soluble in the NaCl of the medium. If the clearing is due to weak acid, an accumulation of acid as the colony grows older causes an opaque zone to appear close to the colony. This zone represents the production of the insoluble di-acid salt by stronger acid. The same effect may be produced by flooding the plate with any dilute mineral or organic acid. If the clear zone changes to an opaque one, weak acid clearing is indicated. If the zone remains clear, the original clearing was due to true hydrolysis of casein, as reported by Hastings.[34] Tested in this way milk agar plates of the butyl organism indicate true hydroysis of casein.

Furthermore, acid clearing is possible only in a medium containing sodium chloride in concentration sufficient to dissolve the mono-acid salt of casein. In the older formulas for nutrient agar, 0.5% NaCl was commonly used and made it necessary to distinguish acid clearing from true hydrolysis of casein. In the present case acid clearing is ruled out because the medium contained no sodium chloride except the traces present in all milk. Such a small amount of sodium chloride cannot induce clearing as proved by the fact that none of the control acid producing anaerobes cleared the medium although all must have produced weak acid and the resulting mono-acid salt of casein. While this method of testing for proteolysis has been condemned by Slobodska-Zaykowoka [35] because of confusion with acid clearing, the objection has been answered, and it seems fair to say that the method is very useful in detecting casein digestion by certain of the butyric acid bacteria. There must also have been action on casein in the tube cultures, although ordinary observation of the curd failed to detect it.

[34] Centralbl. f. Bakteriol., 2, O., 1904, 12, p. 590.
[35] Biochem. Ztschr., 1925, 159, p. 216.

In milk cultures more than 10 to 14 days old there appear white crystalline granules scattered throughout the medium. They have been picked out and found to be calcium citrate with certain phosphate impurities. Complete qualitative and quantitative analyses will be presented later in a separate paper.

Production of Hydrogen Sulfide.—There is no standard method for the detection of hydrogen sulfide.

The one most commonly used is a lead acetate agar method proposed by Kligler [36] and offered by the Committee on Bacteriological Technique [37] as a provisional method. The medium is ordinary beef-peptone agar of P_H 7.4 or 7.6, with 30 Gm. of peptone per liter, instead of the usual 5 grams; to the finished agar an equal part of 0.1% basic lead acetate is added. Stab cultures are made and examined for blackening along the line of growth. The butyl alcohol organisms grow very poorly or not at all in this medium. Two modifications have therefore been made—a change of reaction to P_H 7.0, and the addition of 0.25% glucose—but neither was successful in supporting good growth.

It seemed possible that the lead acetate in the medium was the cause of poor growth. The obvious remedy, then, was to test the cultures after growth by the addition of lead acetate or to test the gas as it came off during growth. Cultures in peptone water were so tested but were negative in all cases, although growth was good. Oatmeal cultures of the butyl organisms were said to have a peculiar odor suggesting hydrogen sulfide. Moist lead acetate paper was hung over active oatmeal cultures. Within 24 hours the paper was tinged with brown by hydrogen sulfide from all of the butyl cultures. In 48 hours the blackening was unmistakable.

An exact duplication of the experiment with corn mash cultures yielded no hydrogen sulfide whatever. The explanation probably lies in the difference between the proteins of oats and corn. Some of the sulfur of the oat kernel appears to be more loosely bound than that in the corn kernel.

In Hall's [28] classification of anaerobes great stress is laid on the ability of the putrefactive anaerobes to blacken brain medium. Brain tissue offers sulfur in its protein for hydrogen sulfide production and iron for its precipitation as iron sulfide. The result is a very striking blackening by iron sulfide. The medium is a sheep or beef brain mash, with the addition of 2% peptone and 0.1% glucose. Hall [19] recommends the glucose as favoring anaerobic growth and the peptone as "hastening and intensifying the blackening." Peptones, especially Bacto peptone, contain appreciable quantities of sulfur and iron and for that reason favor the reaction. Hall [38] in a second paper describes the addition of pieces of sterilized iron to intensify the blackening.

In such a medium the butyl organisms grew very well, but produced no blackening in medium with or without iron. The iron medium did become spotted with dark reddish brown by what appeared to be purely chemical changes. Sterile controls were consistently darkened in proportion to the amount of iron added. Hall in the last mentioned paper has also noted this red iron oxide deposit in some cultures and in some sterile controls. He has observed that the blackening is "perfectly correlated with the production of H_2S in the presence of

[36] Am. J. Pub. Health, 1917, 7, p. 805.
[37] J. Bact., 1919, 4, p. 129.
[38] J. Infect. Dis., 1921, 29, p. 321.

iron." Evidently the butyl organisms cannot liberate hydrogen sulfide from the combined sulfur in brain protein.

To this point the production of hydrogen sulfide has meant its liberation from protein or peptone sources, or more specifically from cystine. Hydrogen sulfide may also result from the reduction of inorganic sulfur compounds. From an investigation of commercial peptones as sources of hydrogen sulfide Tilley[39] concluded that their variable composition made them unreliable in test mediums and he recommended that 0.25% of sodium thiosulfate be added. Following his suggestion Kahn[40] made a study of hydrogen sulfide production by sporulating anaerobes in a thiosulfate medium. The thiosulfate may be added to any medium that supports good growth, preferably one very low in carbohydrate. The brain medium of Hall is satisfactory for the butyric anaerobes and has been used in repeated tests. Two or three drops of 10% lead acetate per tube are not enough to inhibit growth but will detect hydrogen sulfide production. All strains of the butyl organisms produced hydrogen sulfide abundantly from thiosulfate.

TABLE 1

Summary of Hydrogen Sulfide Production as Shown by Lead Acetate Test

Mediums	Cultures										
	1	2	3	4	5	6	7	8	9	10	11
Sulfur from organic source											
Lead acetate agar (P_H 7.4, 7.6 and 7.0, and glucose 0.25%)											
Peptone water											
Synthetic A minus sulfates	0	0	0	0	0	0	0	0	0	0	0
Brain mash, plain and iron											
Corn mash											
Oatmeal mash	+	+	+	+	+	+	+	+	+	+	+
Sulfur from inorganic source											
Synthetic A (sulfate)	0	0	0	0	0	0	0	0	0	0	0
Thiosulfate brain	+	+	+	+	+	+	+	+	+	+	+
Sulfite											
Wilson and Blair agar	0	0	0	0	0	0	0	0	0	0	0
Sulfite brain	+	+	+	+	+	+	+	+	+	+	+

0 indicates negative test.

Recently there has been developed in water work a method for detection of sulfite reducing organisms, particularly anaerobes. Wilson and Blair[41] have pointed out that on anaerobic plates of their medium Cl. welchii, Cl. botulinum and certain other anaerobes produce black colonies, while B. butylicus does not. In such a medium the butyl organisms refused to grow in three successive trials. A control culture of Cl. welchii grew abundantly with typical black colonies. The agar contains 0.6 cc. of 10% NaOH in 100 cc. Such a decidedly alkaline medium may be favorable for pathogenic anaerobes, but it is not so for the butyl group. Accordingly, a series of plates was poured without the NaOH and still no growth could be induced. It is strange that Wilson and Blair[41] found no difficulty with B. butylicus, which must be one of the butyl group of butyric bacteria.

From the sulfite agar plates it was impossible to decide whether the butyl organisms could or could not reduce sulfites. The thiosulfate reduction suggested that the question of sulfite reduction might be

[39] J. Bact., 1923, 8, p. 287.
[40] Ibid., 1925, 10, p. 439.
[41] J. Hyg., 1925, 24, p. 112.

settled in the same way. Accordingly, brain medium was prepared with 0.25% sodium sulfite and two drops of 10% basic lead acetate solution per tube. Again growth was good and all strains of the butyl organism produced hydrogen sulfide.

A summary showing the relation between H_2S production and type of medium is given in table 1.

Indol Production.—It has been customary to test for indol directly on peptone water cultures. But the composition of the peptone is an important factor in the production of indol. It is recommended by the Committee on Bacteriological Technique [37] that, before use, a given peptone be tested for indol with the colon organism.

Bacto and Witte's peptones have been tested; both have been used because they showed some variation as sources of indol. Both the vanillin and Ehrlich's tests were made. All strains of the butyl organism were negative by the direct test after two and five days. To detect small amounts of indol, tests were carried out on distillates by a method recommended by Fellers and Clough.[42] The control culture of B. coli gave a strong positive test from both peptones by both tests. All strains of the butyl organism were negative.

Growth on Blood Agar.—Growth of anaerobic bacteria on blood agar is of interest because, according to McLeod and Gordon,[43] and others, hydrogen peroxide production may be shown, and on blood agar plates hemolysis may also appear.

The butyl organism, like most other anaerobes, produces no catalase as shown by tests with solutions of hydrogen peroxide. According to McLeod and Gordon, then, it should produce a band of green discoloration at the surface of chocolate agar shake cultures, a phenomenon which they assume to be evidence of hydrogen peroxide production. The butyl organism grows well in chocolate agar but does not produce such a green band. Twenty-four hour cultures are distinctly reddened in the depths of the medium, as McLeod and Gordon have noted with the cultures of Cl. welchii, Cl. chauvoei, and Vibrion septique. They speak of the redness as the "color of the blood pigment in its reduced state." After 48 to 72 hours the entire medium becomes a dark opaque greenish brown. McLeod and Gordon have observed that organic acids, particularly butyric and lactic, in concentrations of 1 to 5%, will cause a generalized discoloration of chocolate agar. The butyl organism in a glucose medium, of course, produces butyric acid; accumulation of acid then accounts for the general discoloration in old cultures.

Blood agar cultures give evidence of the production of hemolysins and of the type of hemolysis. Brown [20] in a recent study of anaerobic bacteria found that the majority produce beta hemolysis. None that he studied was of the alpha type and only a few of the gamma type.

[42] J. Bact., 1925, 10, p. 105.
[43] J. Path. & Bact., 1923, 26, p. 332.

On unheated blood agar plates none of the butyl strains showed the least sign of hemolysis. The butyl organism then produces gamma type colonies on blood agar. Anaerobic incubation of blood agar changed its color from a clear red to a dull reddish brown. On return to aerobic conditions, the control plates partly regained their color, but they never again were bright red. The culture plates were opaque and grayish brown from butyric acid and did not change on contact with air.

Fermentation of Corn Mash.—The most distinctive feature of the butyl organism is its fermentation of corn mash, a fermentation of growing industrial importance.

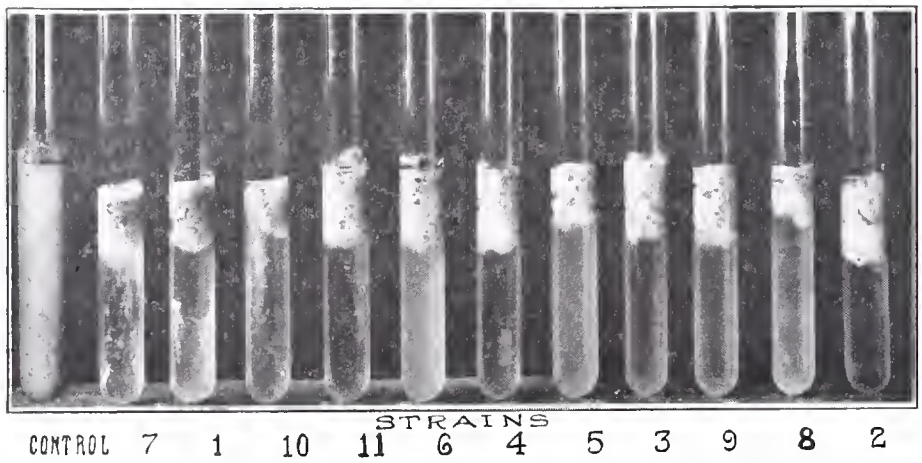


Fig. 3.—Growth of butyl organisms (11 strains) in corn mash. (Description of strains on page 459.)

The mash for either large scale production or laboratory cultures consists of 5 to 10% corn meal in water. In this the butyl organism multiplies very rapidly until within 24 hours it numbers between 1 and 2 billion cells per cc. During that time profound chemical changes take place. The original medium is a thick starch gel. Fermentation produces in it the change illustrated in text figure 3. The mash is rapidly liquefied, much gas is produced, and the insoluble corn particles are raised as a head. The whole fermentation is an example of a delicate balance of biological processes. In the first place the organism produces a very active diastase which converts the corn starch into reducing sugar. Simultaneously the fermentation of the sugar leads to a rapid rise in acidity. At some time between 15 and 21 hours after inoculation, there is normally a sudden drop in acidity. This is accounted for by the conversion of acid into the neutral solvents, acetone, butyl alcohol and ethyl alcohol—a conversion more rapid than the parallel acid production. As the activity slackens toward the end of the fermentation there is a gradual secondary rise in acidity. During the whole fermentation proteolytic action is slowly increasing the proportion of the soluble nitrogen. It is probable that proteolysis plays an important part by its buffer effect. The gases evolved are carbon dioxide and hydrogen in the proportion of 60 to 40 by volume.

Many quantitative studies have been made of the major and minor products of fermentation. In this laboratory an attempt has been made to correlate the biological changes with chemical changes throughout the fermentation. A complete report of that study will be presented in a separate paper. At this time a short summary of fermentation products will be of interest.

PRODUCTS OF FERMENTATION OF 100 GRAMS STARCH OR 136 GRAMS OF DRY CORN MEAL

		Grams
Total solvents		35.8
Acetone	10.56	
Butyl alcohol	20.76	
Ethyl alcohol	4.47	
Gas		61.5
Carbon dioxide	59.60	
Hydrogen	1.90	
Residual acid		2.7
Total		100.0

PRODUCTS FROM PROTEINS IN 100 GRAMS OF DRY CORN MEAL

	Before Fermentation, Grams	After Fermentaion, Grams
Total nitrogen	1.52	1.49
Insoluble nitrogen	1.28	0.64
Soluble nitrogen	0.24	0.85
Protein	0.13	0.52
Nonprotein	0.11	0.33
Soluble amino nitrogen	0.04	0.24

Fermentation of Other Carbohydrates.—Potato: Both Omeliansky [44] and Winogradsky [45] have reported that "Cl. pasteurianum on potato without calcium carbonate gives a strong odor of butyric acid. The colonies on potato appear yellowish and measure never to exceed a millimeter in width and as much in height." Neither mentioned any disintegration of the potato tissue. The butyl organisms with their strong diastatic action and their rapid conversion of acid into solvents might be expected to have an entirely different effect on potato. Such is the case.

Plain potato slants and potato slants plus calcium carbonate were inoculated and incubated in anaerobic jars at 37 C. Examination after 48 hours showed a creamy yellow surface growth of slimy consistency. Gas bubbles swelled and cracked the layer of growth and even seemed to form within the potato tissue. The potato itself was wrinkled and shrunken. A diffuse yellow pigment showed plainly both on the slant and in the liquid below. No odor of butyric acid could be detected in either plain or calcium carbonate cultures. Rather a sweet solvent odor characterized all strains.

Later examination showed the same general characteristics intensified. The potato tissue had disintegrated to a slimy mass. The yellow pigment and solvent odors were strong and a test with iodine showed no remaining starch. Upon microscopic examination only a mass of spores and tissue debris was found.

[44] Arch. d. Sc. biol., 1915, 19, p. 209.
[45] Ibid., 1895, 3, p. 297.

After a two months' incubation, the last half of which was aerobic and at room temperature, all of the calcium carbonate cultures of the butyl organisms had produced hard white granules apparently like those in milk cultures. It seems probable that they also were a calcium compound but they were not identified chemically.

In Synthetic Medium: The value of fermentation reactions in the description of bacterial species is beyond question. The only chance for dispute lies in the acceptance of the true criteria of fermentation. With aerobic and facultative bacteria the production of acid and gas are generally believed to indicate carbohydrate fermentation. There are, however, cases of fermentation with acid and little or no gas, and the converse.

In the case of anaerobic bacteria there is more uncertainty in the interpretation of fermentation tests. Hall [38] in a review of criteria of anaerobic fermentation rejects both CO_2 production and titratable acidity as infallible evidence of fermentation, although he looks upon both tests as good indications. He concludes that the best single test of fermentation is an increase in H ion concentration. The degree of change in true acidity may not have a direct relation to the degree of fermentation. Brown in a recent paper on anaerobic bacteria has chosen as the most logical criterion, the "disappearance of the test substance." That is undoubtedly the best evidence of fermentation. Unfortunately, its application is limited to those carbohydrates for which there are quantitative methods of analysis; that is, to the reducing sugars. For the others, one must judge fermentation from the products formed, such as gas, acid, and any special products that can be detected. The eight reducing sugars and also sucrose have been measured quantitatively before and after fermentation by the Shaffer-Hartmann micro method as modified by Stiles, Peterson, and Fred.[46] The fermentability of other carbohydrates is judged from production of gas, titratable acid, acetone, and acetyl methyl carbinol.

All fermentation reactions were studied in synthetic medium A containing about 1% of carbohydrate. The sugars were sterilized separately in water and were added aseptically to the double strength medium. The choice of inoculum brought up new problems because anaerobes are commonly grown in a carbohydrate medium. If the inoculum is taken from a young culture, it will carry some carbohydrate; if it is taken from a culture at the end of fermentation, it may produce a weak culture. Preliminary tests with glucose showed that the inoculum from a 48 hour old culture of the butyl organism in 1% glucose synthetic medium A is too weak to give consistent growth. The same is true of suspensions of 48 hour cultures grown on beef-peptone glucose agar. A satisfactory inoculum

[46] J. Bact., 1926.

is one of 24 hour corn mash culture in 1:40 ratio. Such an inoculum carries enough carbohydrate to support a slight fermentation, but it is of no consequence when the criterion is the disappearance of the test substance. In other cases, where the products of fermentation are measured, fermentation of the inoculum must be taken into account and its products subtracted from the combined fermentation products in the test cultures. The advantages of a vigorous inoculum entirely outweigh the disadvantage of the very small amount of carbohydrate it may carry.

The fermentations of pentoses, hexoses, and disaccharides will be considered together because quantitative sugar analyses have been made of all of these compounds. To insure good growth, the fermentation tubes were incubated in anaerobic jars. Fermentation of a 1% carbohydrate medium by all strains of the butyl organism was rapid and was practically complete in 3 to 5 days. Analyses were not made until 48 hours after the cultures had ceased gassing. Table 2 presents the data on fermentations of nine reducing sugars and sucrose calculated as glucose. In each case the results are expressed as percentage of sugar fermented.

TABLE 2

THE PERCENTAGE FERMENTATION OF VARIOUS SUGARS OF ABOUT 1% CONCENTRATION

Sugars	Cultures										
	1	2	3	4	5	6	7	8	9	10	11
Pentoses											
Arabinose	95	96	95	96	95	95	97	95	97	94	95
Xylose	95	86	89	86	88	86	96	91	89	93	92
Methyl Pentose											
Rhamnose	No test	16	16	11	14	18	No test	12	11	11	8
Hexoses											
Glucose	93	98	97	98	98	98	99	97	98	98	98
Galactose	95	94	95	97	95	95	95	97	97	95	95
Mannose	95	95	95	97	95	95	95	97	95	97	95
Fructose	95	95	96	96	97	95	95	96	95	96	95
Disaccharides											
Sucrose	97	95	95	95	95	95	92	97	95	95	95
Maltose	95	95	95	95	95	95	95	95	95	95	95
Lactose	93	92	92	95	95	95	95	83	92	95	95

The figures of the table show that under the conditions used, the reducing sugars in 1% concentration are almost completely utilized by the butyl organism. There is no marked difference between the degree of fermentation of the pentoses, hexoses, and disaccharides. Rhamnose, a methyl pentose, is only slightly attacked.

The great majority of carbohydrate fermentations must be studied indirectly. Of the 33 compounds used in this investigation, only 10 could be measured directly. For the others, evidence of fermentation has been collected from a number of sources, no one of which alone is significant. When there is active fermentation, the combined evidence is convincing. When fermentation is poor, there is chance for doubt. For a compound to be classed as fermentable, two or more tests of its fermentation products should be unquestionably positive.

Production of gas, titratable acid, acetone, and acetyl methyl carbinol have been studied in a number of carbohydrate fermentations. Fermentation by the butyl organism is always associated with gas production.

However, gas may be formed in sugar free mediums by certain proteolytic anaerobes. Henry [33] has observed that such gas production is slow and continues over a long period of time, whereas the fermentation of carbohydrates is rapid at first and stops when the substance is exhausted. He considers early gas production by anaerobes a valuable test of fermentation. Kahn [30] has also laid emphasis on gas production. He

TABLE 3

Total Gas Production During Fermentation of Various Carbohydrates, Glucoside and Alcohols After 6 Days at 37 C.

	Rise of Plugs (Cm.) During Fermentation of 10 Cc. Cultures: Strains										
	1	2	3	4	5	6	7	8	9	10	11
Methyl Pentose											
Rhamnose	4.0	5.5	6.0	3.0	4.0	6.5	2.5	3.5	2.0	2.0	3.0
Hexose											
Glucose	28.0	30.5	27.5	27.0	30.0	30.0	23.5	24.0	29.5	35.0	30.0
Disaccharides											
Trehalose	7.5	9.5	7.5	9.5	6.5	5.0	7.0	10.0	7.5	13.5	6.0
Melibiose	3.0	4.0	3.5	3.5	5.0	3.0	4.0	3.5	3.0	3.5	3.5
Trisaccharides											
Raffinose	11.5	13.5	17.5	11.5	11.0	10.5	14.5	12.5	10.5	12.0	15.0
Melezitose	26.5	20.0	17.5	17.0	24.0	22.5	18.5	17.5	26.0	18.0	17.0
Polysaccharides											
Starch	23.5	25.5	27.5	24.0	23.5	21.5	24.5	17.5	22.0	24.0	25.0
Dextrin	21.5	23.0	27.5	30.5	30.5	27.5	20.0	28.0	31.5	29.0	32.5
Inulin	31.0	26.5	30.8	32.0	30.7	22.0	34.0	27.0	27.0	25.0	30.0
Glycogen	20.0	22.5	21.0	22.5	25.0	22.0	21.5	22.0	26.5	25.5	25.5
Alcohols											
Glycerol	4.0	3.0	4.0	3.0	2.0	3.0	3.0	2.5	3.5	3.5	2.0
d-Mannitol	29.0	25.5	37.0	33.0	32.0	25.0	37.0	32.5	23.0	35.0	30.5
Dulcitol	3.0	3.5	4.5	4.0	4.0	4.5	3.0	5.0	5.0	3.5	5.0
l-Arabitol	2.0	5.0	3.5	3.5	4.0	3.0	2.5	4.0	2.5	5.5	3.0
Perseitol	2.5	5.0	5.5	2.5	2.5	2.5	3.0	6.5	3.0	4.5	3.0
Lactositol	4.5	8.0	5.5	5.0	7.5	6.0	7.0	5.0	8.0	6.5	6.5
Sorbitol	2.0	4.5	4.5	4.0	4.0	2.0	4.0	3.0	2.5	2.5	2.5
Erythritol	2.0	6.5	3.0	3.5	5.0	3.0	3.0	6.0	3.5	4.0	2.5
Adonitol	8.5	10.5	7.0	5.0	11.5	7.5	6.0	6.0	10.5	5.0	6.0
Inositol	2.5	5.0	4.0	4.0	3.5	3.0	2.5	4.0	4.5	5.0	1.0
Quercitol	2.0	6.5	6.5	2.5	4.0	2.0	2.5	3.0	5.0	4.5	3.0
Glucosides											
α-methyl glucoside	32.0	30.5	29.0	28.0	26.0	36.0	36.0	25.0	26.0	27.0	26.5
Salicin	18.5	23.0	21.5	22.0	22.5	17.0	18.0	17.5	21.0	16.5	21.5
Amygdalin	7.5	10.0	10.0	13.0	9.0	10.5	12.0	12.5	11.0	11.0	12.0
Esculin	13.0	14.0	15.5	16.0	19.5	16.0	14.5	15.5	19.0	16.5	15.5

has measured gas by the rise of vaseline plugs during the incubation of cultures. The vaseline serves also as an anaerobic seal, a method originally used by Loewe and Strauss.[47]

In the present investigation vaseline seals have proved very helpful. They have furnished the gas data in table 3. The figures given are approximate only, because of slight variations in size of the tubes, weight of plugs, and measurements of rise. Fermentation of the inoculum alone in control tubes gave 3 to 5 cm. of gas, all formed within the first 24 to 36 hours after inoculation. Gas production in positive fermentations continued over a longer period and was decidedly greater. The figures in the table represent the net gas production with the controls subtracted. Glucose which was analyzed with the previous group of fermentations is included here for the sake of comparison. From the data it is not

[47] J. Infect. Dis., 1920, 27, p. 250.

hard to choose the most fermentable compounds. But what shall be said about those with little gas? The gas may mean slight fermentation or it may come from other constituents of the medium. The decision must wait for support from the other indirect tests.

Acetone is a characteristic product of the butyl fermentation and one which can be measured quantitatively. The analyses are presented in table 4. The values for glucose are low compared with those of Peterson, Fred and Schmidt[48] for 3% glucose. A further study was, therefore, made to see whether the con-

TABLE 4

THE PRODUCTION OF ACETONE FROM VARIOUS CARBOHYDRATES

	In 100 Cc. of Culture			
	Strain 7		Strain 5	
Carbohydrates	Acetone, Mg.	N/10 Acid, Cc.	Acetone, Mg.	N/10 Acid, Cc.
Glucose	19.8	41.8	36.0	41.0
Rhamnose	1.4	12.0	3.4	12.0
Raffinose	0.9	25.0	0.9	21.0
Melezitose	30.5	55.0	36.0	54.0
Starch	30.7	42.0	39.4	40.0
Dextrin	33.3	44.0	34.0	44.0
Inulin	20.4	49.5	29.0	50.6
Glycogen	8.5	51.0	15.2	47.0
Salicin	12.0	44.5	8.8	42.5
α-methyl glucoside	60.0	18.0	44.0	28.0
Amygdalin	18.0	31.0	12.0	11.0
Esculin	....	19.0	6.0	16.0
Mannitol	8.2	43.5	20.4	44.0
Glycerol	1.1	9.0	1.4	8.0

TABLE 5

THE PRODUCTION OF SOLVENTS AS RELATED TO CONCENTRATION OF CARBOHYDRATES

	For 100 Cc. of Culture			
	Glucose		Starch	
	1%	2%	1%	2%
Carbohydrate fermented, Gm.	0.92	1.80	1.00	2.00
Titratable acid, Cc. N/10	27.0	33.0	36.0	23.0
Solvents, Gm.	0.178	0.480	0.195	0.426
Acetone, Gm.	0.044	0.155	0.037	0.110
Percentage acetone in solvents	25.0	32.2	18.7	25.8
Percentage of carbohydrate converted into solvents.	18.6	26.8	19.5	21.3
acetone..	4.6	8.3	3.7	5.5

centration of carbohydrate affected the production of acetone. Cultures of 1 and 2% carbohydrate in synthetic medium A were fermented by strain 7. The results with glucose and starch are presented in table 5. It is evident that the yield of acetone increases with an increase in concentration of carbohydrate. The residual acidity of the fermentation is the same, however. One may consider, then, that in a 1% concentration the greater part of the carbohydrate is accounted for in the acid, whereas in a 2% medium more than twice as much carbohydrate is available for solvent production. In table 4, then, those carbohydrates which gave yields comparable to those of glucose or starch may be considered fermentable.

The titration of acid in fermentable mediums may be considered a third indirect test of fermentation. In this particular fermentation it is admittedly a poor

[48] J. Biol. Chem., 1924, 60, p. 627.

measure of the changes because a large part of the sugar utilized has appeared as neutral solvents. However, the titrations are of some value and are presented in table 4 with the acetone data, and in table 6. The figures are corrected for acidity of the medium and of the inoculum.

Acetyl Methyl Carbinol: The production of acetyl methyl carbinol is generally associated with glucose fermentation by members of the colon-aerogenes group. Its detection by the Voges-Proskauer [49] test is a routine procedure of water analysis in the differentiation of the true colon organism from the aerogenes type. The original Voges-Proskauer test applied only to the eosin pink coloration of glucose broth cultures on the addition of strong alkali. The chemistry of the reaction has been studied by Harden and Norris [50] who showed the color to be due to

TABLE 6

Total Titratable Acid Produced in the Fermentation of Certain Carbohydrates

	Cc. N/10 Acid in 10 Cc. of Culture: Strains										
	1	2	3	4	5	6	7	8	9	10	11
Glucose	5.0	4.6	4.2	3.8	4.6	5.0	4.4	5.2	4.0	5.0	4.8
Trehalose	3.0	4.6	3.6	4.2	4.2	5.2	4.0	2.4	5.2	4.8	3.0
Melibiose	1.6	1.2	2.2	1.2	1.2	1.2	1.6	1.4	2.2	1.2	1.2
Raffinose	3.2	3.4	4.4	3.6	3.4	4.0	3.4	3.6	3.2	3.6	4.2
Starch	5.0	4.6	4.6	4.8	5.0	5.0	4.6	5.0	4.8	4.6	5.2
Dextrin	3.6	2.7	2.6	3.6	2.8	3.6	3.7	2.6	3.1	2.9	3.3
Inulin	3.3	3.5	3.2	3.8	3.7	2.5	4.6	3.7	3.3	2.7	3.6
Glycerol	0.1	1.1	0.6	0.2	0.1	0.3	0.5	0.5	0.7	0.3	0.2
Mannitol	2.8	3.4	2.6	3.1	2.0	3.5	3.3	3.2	3.4	2.7	2.8
Quercitol	1.4	2.4	2.6	1.8	1.8	2.4	2.0	2.2	2.0	2.2	2.0
Dulcitol	1.8	3.0	3.0	1.8	2.8	2.2	2.2	1.6	2.2	2.6	2.4
Arabitol	1.4	2.6	2.2	2.6	1.8	3.0	2.0	2.8	2.2	3.0	1.6
Perseitol	1.2	2.4	2.2	2.0	2.0	1.8	2.0	1.8	2.4	2.2	2.0
Lactositol	3.2	2.6	3.2	2.6	2.8	2.8	2.4	2.4	3.0	2.8	2.4
Sorbitol	1.4	2.2	2.6	3.0	2.0	3.8	2.0	3.0	1.8	2.2	2.6
Erythritol	1.8	2.6	2.2	2.4	2.8	2.0	3.0	2.4	2.0	2.6	2.2
Inositol	1.7	1.4	1.1	1.0	1.1	1.0	1.5	0.9	1.2	1.1	0.8

acetyl methyl carbinol and potassium hydroxide in the presence of peptone. Since then the same compound has been found among the fermentation products of other sugars. In view of the fact that the Voges-Proskauer reaction applied to glucose only, Levine [51] suggests that the same test on other sugars be called the "Carbinol Test."

There are many references to acetyl methyl carbinol production by other than the colon-aerogenes organisms; for instance, by B. subtilis, B. proteus vulgatus, staphylococci, and yeasts. As far as the literature has been reviewed there is record of acetyl methyl carbinol production by only one facultative anaerobe and by no obligate anaerobes. The compound is probably an acyloin synthesis product formed by the

[49] Ztschr. f. Hyg., 1898, 28, p. 20.
[50] Proc. Roy. Soc., 1911, 84, p. 492.
[51] J. Bact., 1916, 1, p. 153.

action of the enzyme, carboligase, on acetaldehyde. Berthelot[52] has shown that B. aminophilus, valuable for symbiotic growth with anaerobes, produces acetyl methyl carbinol and pyruvic acid. Both substances are reducing agents and promote anaerobic growth.

It is not surprising, then, to find that the anaerobic butyl organisms produce acetyl methyl carbinol from a variety of carbohydrates. The 33 carbohydrates grouped with respect to their yield of acetyl methyl carbinol follow.

Acetyl Methyl Carbinol

Positive	Negative
Starch, glucose, +++	Dulcitol, sorbitol, melibiose, quercitol, esculin (brown), glycerol, arabitol, perseitol, lactositol, erythritol, inositol and adonitol
Dextrin, mannose, sucrose, maltose, arabinose, ++	
Lactose, inulin, salicin, xylose, rhamnose, galactose, fructose, raffinose, melezitose, glycogen and α methyl glucoside, +	
Trehalose, amygdalin and mannitol, ±	

The tests were made on 7 day cultures with equal quantities of 10% KOH. Color was developed within 18 to 24 hours and faded gradually. The test is crude and unsatisfactory in many ways. Lemoigne[53] reports that it lacks sensitiveness and gives uncertain results. When it is strongly positive, the results are decisive. But, on the other hand, negative results are meaningless.

The significance of the production of acetyl methyl carbinol is the subject of further study.

Nitrate and Nitrite Reduction.—Nitrate reduction has been the subject of considerable study by the Committee on Bacteriological Technique. Reduction is not a constant characteristic of a species and is not always shown on standard nitrate broth, even by known nitrate reducers. Consequently, when all strains of the butyl organisms were found negative by both Trommsdorf's and the sulphanilic acid tests, it was not safe to conclude that under all conditions they were unable to reduce nitrates. The investigations of the Committee point out that absence of nitrite may mean: poor growth, conversion of nitrite into ammonia as fast as produced, assimilation of nitrite-nitrogen as fast as formed, or actual inability to reduce nitrates. In all doubtful cases, it is recommended that medium be modified to insure good growth by "increasing or decreasing the amount of peptone, altering the reaction, or adding some readily available carbohydrate. The appearance of nitrite in any medium whatsoever should be regarded as nitrate reduction."

In the present case the reaction, P_H 7.0, was known to be favorable. The best modification, therefore, seemed to be the addition of a carbohydrate to favor anaerobic growth. One half % of glucose was added to the usual nitrate broth. It allowed vigorous growth but no reduction of nitrate. It is probable that the glucose was used in preference to the nitrate as a source of oxygen.

[52] Compt. rend. Acad. d. sc., 1923, 176, p. 1929.
[53] Compt. rend. Acad. d. sc., 1920, 170, p. 131.

As a final resort for those organisms that are nitrate-negative and yet are able to grow, the Committee recommends the following mediums: Nitrate-peptone agar or broth; peptone agar or broth without nitrate; synthetic nitrate medium (KNO_3 1.0 gram., K_2HPO_4 0.5 gm., $CaCl_2$ 0.5 gm., glucose 10 gm., distilled water, 1,000 cc.); broth or agar with 2 p.p.m. potassium nitrite. The first 3 mediums are to be tested for ammonia by the Thomas test, using 1 cc. each of 5% phenol, and of a solution of sodium hypochlorite (1% available chlorine), and the third and fourth are to be tested for nitrites by sulfanilic acid and α-napthylamine.

All strains of butyl organisms have been repeatedly put through this series of tests. Growth is fairly good in nitrate peptone broth, but there has never been any reduction nor any decided test for ammonia. The faint blue color that sometimes developed, fluctuated and was no more than the same test showed in uninoculated controls. In the broth without nitrate there was no turbidity or other sign of growth. No ammonia was produced in it. The synthetic nitrate medium was not favorable. Although there was scanty growth, neither ammonia nor nitrites were produced in it. The nitrite in the potassium nitrite medium was destroyed by all strains. Growth was very irregular so that sometimes one, sometimes another strain failed. However, the tests have been repeated enough to justify the conclusion that all strains are able to destroy nitrites under favorable conditions. Since corn mash is a far better medium than beef peptone broth for the growth of the butyl organism, it seemed that nitrates might be reduced in it. Mash with 0.1%•KNO_3 was tested at the usual intervals for ten days, but there was no reduction whatever. Corn mash with 2 p.p.m. of KNO_2 regularly gave reduction—a further proof of the special ability to reduce nitrites.

The butyl organisms are acid producing. It seemed possible that acids alone were responsible for destruction of nitrites. Tubes of nitrite broth were, therefore, treated with varying mixtures and concentrations of N/1 butyric and acetic acids. The tubes were incubated and tested as were the previous cultures. In the tubes of maximum acidity, probably 5 to 2.5 times more acid than a culture of the organism could ever be, and especially in such tubes containing powdered zinc, there was action upon the nitrites in 4 to 5 days and total destruction within 10 days' time. In tubes with acidity comparable to that in cultures, nitrites were still present after 10 days. It appears that the bacteria destroyed the nitrites by some mechanism other than acidity, for they completed their work within 24 to 48 hours at a relatively low acidity. It is not apparent whether they produce a specific reducing enzyme, or whether their active hydrogen is responsible.

It has been observed that the butyl organism grows fairly well in nitrate broth, whereas it does not grow in plain broth. Such observations recall those of Winogradsky,[45] and of Beijerinck and Van Delden.[54] The former found no turbidity of his Cl. pasteurianum in plain broth and used the fact as a criterion of purity. The latter made the statement that potassium nitrate stimulates the growth of B. granulobacter pectinovorum.

Crystal Violet.—Certain dyes, particularly the triphenyl methane group of which crystal violet is a member, have inhibitory effects upon bacteria. Since 1912 Churchman [55] has studied what he calls "bacteriostasis" in mediums containing dyes. He and other investigators have found that microorganisms differ in their tolerance for dyes and that some dyes are more injurious to certain species of bacteria than to others. It is well known that the triphenyl methane dyes are more inhibitory for the gram-positive organisms than for the gram-negative. B. subtilis, for example, is inhibited in dilution 1 : 250,000 of crystal violet, while B. coli can thrive in dilution 1 : 100,000 in broth. In fact, 1 : 100,000 crystal violet is being used (Hall and Ellefson [56]) in some water work to exclude gram-positive spore formers, both aerobic and anaerobic, and at the same time to allow growth of any colon organisms there may be. In general the anaerobic spore forming bacteria, although they are gram positive in young cultures, are far more resistant to dyes than are the aerobic gram-positive organisms. Hall presented evidence of this fact from a study of 33 strains of anaerobic spore formers. Fourteen were able to grow in concentration of crystal violet as high as 1 : 10,000.

The tolerance of anaerobes for the triphenyl methane dyes may be of great advantage in their isolation. Hall [57] first advocated separation of anaerobes by successive transfers through gentian violet broth at a concentration of 1 : 100,000, sufficient to kill contaminating aerobic spore formers. The method has not yet been tried in this laboratory in an actual isolation. Many successful transfers have been made, however, from both young and old cultures in crystal violet medium. They show no weakness even when taken from 1 : 10,000 crystal violet. A few transfers at 1 : 20,000 or even 1 : 30,000 at P_H 6.0 would certainly eliminate gram-positive organisms, spore formnig, and nonspore forming, and probably, also, many of the gram-negative organisms. There might still be other anaerobes which would require other means of separation. The use of crystal violet followed by plating and picking of colonies would seem to be a rapid and simple method of isolation.

Crystal violet has not been useful in separating strains of the butyl organisms as will be seen in table 7. In general they show a greater tolerance for the dye than do the majority of anaerobes. However, the type of medium in which the dye is presented must be considered. A solid or semisolid medium allows growth at higher concentrations than does a liquid. In a preliminary experiment with two strains, a rice mash allowed growth in 1 : 5,000 dilutions. But the rice particles

[54] Verhandel. d. Koninkl. Akad. v. Wetenschappen, 1904, 6, p. 462.
[55] Stain Technology, 1926, 1, p. 27.
[56] J. Bact., 1918, 3, p. 329.
[57] J. Am. M. A., 1919, 72, p. 274.

evidently absorbed the dye. The bacteria, then, were not actually growing in 1 : 5,000 crystal violet.

The data presented in table 7 are based on growth in synthetic medium A with concentrations of crystal violet from 1 : 100,000 to 1 : 10,000. The organisms grow very well in this medium, gassing vigorously, producing much slime, and decolorizing the dye. Glucose (Merck, C. P.) has been used in the medium so that there can be no question of reduction by the sulfite impurities in the sugar.

The hydrogen ion concentration is a large factor in the penetration of dyes into cells. Irwin[58] has shown that penetration of dye into cells of Nitella increases with increase of alkalinity. For a difference of P_H 6.6 to 7.0, a small change, he found a fourfold increase in penetration. Organisms should, therefore, tolerate greater concentration of dye in an acid medium than in a neutral or alkaline one. Table 7 shows this to be so with butyl bacteria. At P_H 7.0 only strain 5 was able to grow at 1 : 10,000 concentration of crystal violet. The other strains were inhibited at 1 : 30,000 or 1 : 20,000 dilutions. The difference between 1 : 10,000 and

TABLE 7

Growth in Crystal Violet Mediums of Different Hydrogen-Ion Concentrations

	Concentration of Crystal Violet in Synthetic Medium A									
	1:100,000	1:50,000	1:30,000		1:20,000		1:15,000		1:10,000	
Strain	P_H 6.0 and 7.0	P_H 6.0 and 7.0	6.0–P_H–7.0		6.0–P_H–7.0		6.0–P_H–7.0		6.0–P_H–7.0	
1	++	++	++	++	++	+ 0	++	0	++	0
2	++	++	++	++	++	0	+ 0	0	0	0
3	++	++	++	+ 0	++	+ 0	++	0	++	0
4	++	++	++	++	++	++	++	0	++	0
5	++	++	++	++	++	++	++	++	++	++
6	++	++	++	++	++	+ 0	++	0	++	0
7	++	++	++	++	++	+ 0	++	0	++	0
8	++	++	0	++	++	0	0	0	0	0
9	++	++	++	++	++	++	++	+ 0	++	0
10	++	++	++	++	0	+ 0	++	0	0	0
11	++	++	++	++	++	++	++	0	++	0

++ indicates growth and complete reduction; + 0, growth but no reduction; 0, no growth and no reduction.

1 : 20- or 30,000 is hardly enough to set off one strain from another, although it was a consistent difference in three successive tests with triplicate tubes. At P_H 6.0 strain 5 is no different from the others, for practically all strains were able to grow in 1 : 10,000 dilution.

In a recent paper Churchman[55] states that gentian violet and related dyes inhibit sporulation of bacteria and cites his observations on B. subtilis. Our observations show that anaerobic spore-formers, at least those of the butyl group, are not so affected. Five strains of the butyl organism in cultures 15 days' old have shown normal numbers of spores in concentrations of crystal violet from 1 : 100,000 down to 1 : 10,000.

CONCLUSIONS

The butyl organism is a prominent member of the low acid producing, and high alcohol producing group of anaerobic butyric acid bacteria.

[58] J. Gen. Physiol., 1922, 5, p. 727.

PLATE 1

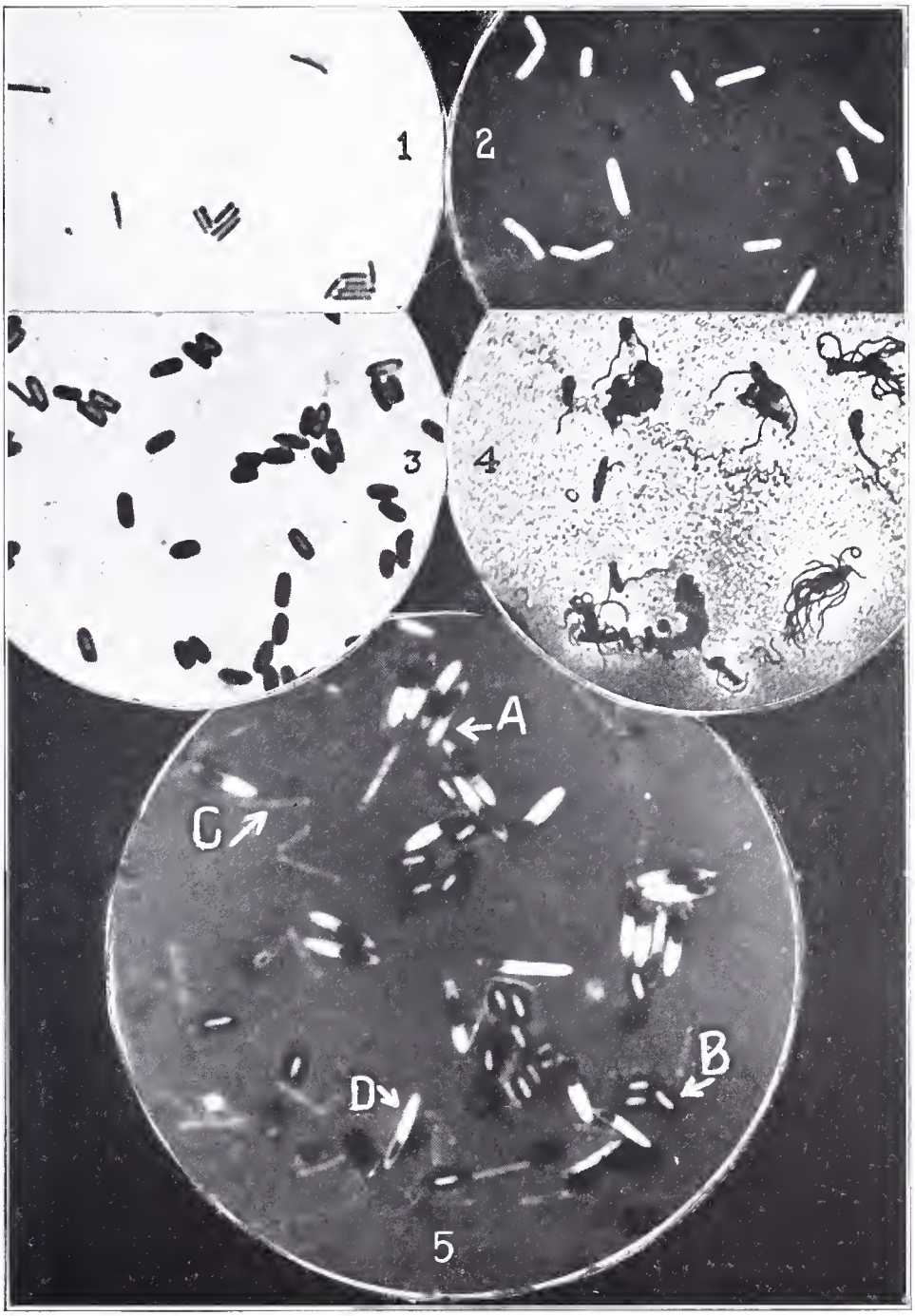


Fig. 1.—Strain 7: Vegetative cells of 2 hour culture in corn mash. "Rafting." Carbol fuchsin.

Fig. 2.—Strain 7: Vegetative cells of 24 hour culture in corn mash. × 1050, Nigrosin.

Fig. 3.—Strain 7: Spores from surface colony on beef peptone glucose agar. 7 days old. Carbol fuchsin.

Fig. 4.—Strain 7: Flagella on cells from 24 hour surface colony on beef peptone glucose agar. × 1050, Plimmer-Paine method.

Fig. 5.—Sporulation of Strain 5. From surface colony on beef peptone glucose agar 7 days old. × 1200, Dorner method. A. Sporangia with mature spores. B. Free spores with halo of nigrosin. C. Dead vegetative cells. D. Young clostridium.

Morphologically it is characterized as a motile gram-positive bacillus, bearing granulose and swelling to clostridial form at sporulation. Its large oval spores appear in excentric or subterminal position.

The butyl organism is extensively proteolytic. Gelatin is rapidly hydrolyzed as shown by a correlation of physical and chemical tests. Coagulated albumin is attacked in any medium supporting growth of the organism. A part of the casein is hydrolyzed, but not enough to be apparent in tube cultures in milk. Blood serum and brain tissue, both complex animal proteins, are not visibly changed. Blood is not hemolyzed. Hydrogen sulfide from an organic source was found from the proteins of oats only. As inorganic sources, sodium thiosulfate and sodium sulfite were available.

Fermentation of corn mash by the butyl organism is important as an industrial process and as a characterization of the organism. The major products are the solvents, acetone, butyl and ethyl alcohols, the acids, butyric and acetic, and the gases, hydrogen and carbon dioxide.

A great variety of carbohydrates are fermented: arabinose, xylose, rhamnose, glucose, galactose, mannose, fructose, sucrose, maltose, lactose, raffinose, melezitose, starch, dextrin, inulin, glycogen, d-mannitol, α methyl glucoside salicin, esculin (?), amygdalin (?) and trehalose(?). The products are essentially the same as those of the corn mash fermentation. There is some evidence that the balance of products is different with certain of these compounds. The following are not fermented: melibiose, dulcitol, d-arabitol, perseitol, lactositol, sorbitol, erythritol, adonitol, inositol, quercitol, glycerol, and cellulose.

Acetyl methyl carbinol has been detected in many fermentations by the butyl organism.

Nitrates are not reduced by the butyl organism, but nitrites are rapidly and completely destroyed. The mechanism of nitrite destruction is not known; it does not appear to be simple reduction to ammonia, nor yet the results of action of the acids.

Slight variations between the 11 strains of the butyl organism studied in this laboratory do not warrant separation into different types.

In view of the fact that no organism hitherto described possesses the primary characteristics of the butyl organism, none of the names of the earlier butyl alcohol producing organisms is applicable. It is, therefore, proposed that the acetone butyl alcohol organism be called Clostridium acetobutylicum (Weizmann).

THE ISOLATION OF SUBSTANCES WITH IMMUNE PROPERTIES

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THE PURIFICATION OF DIPHTHERIA ANTITOXIN

The need for the purification and concentration of diphtheria antitoxin was perhaps most keenly felt during the first ten years of its production, when serums of satisfactory titer were not universally available. During these years, it was found that the antitoxic potency of a serum may be concentrated into its pseudoglobulin fraction. Many methods have been developed since then for the simplification of this concentration.[1] These have been practically valuable for salvaging low

TABLE 1

COMPARISON OF THE DEGREE OF CONCENTRATION OF DIPHTHERIA ANTITOXIN WHICH MAY BE SECURED BY DIFFERENT METHODS

	Mg. of Nitrogen Associated With One Unit	Degree of Concentration: Mg. of Nitrogen per Unit Original Serum / Mg. of Nitrogen per Unit Final Preparation (approximate values)
Untreated plasma	0.007 – 0.07	1
Early salt precipitation: methods (Gibson [1]), "insolubilization" (Besredka [1]), nonspecific adsorption [2]	0.016 – 0.02	2 – 3
Improved salt precipitation: methods (Heinemannn [1]), electrodialysis (Ruppel [1])	0.013	3 – 5
Isoelectric precipitation [3]	0.003	12 – 15
Specific precipitation with diphtheria toxin [4], solutions of the precipitate	0.002 – 0.005	10 – 25
Extracts of the precipitate	0.0005 – 0.002	25 – 100
Antityphoid serum	$6 - 11 \times 10^{-4}$	1
Best antibody preparation [5]	0.045×10^{-4}	125 – 250
Antipneumococcus serum	4.8×10^{-7}	1
Best antibody preparation [6]	0.0056×10^{-7}	860
Amboceptor serum	0.01	1
Best hemolysin preparation [7]	0.000,007	1500

Received for publication, July 23, 1926.

[1] Gibson: J. Biol. Chem., 1906, 1, p. 161, with review of the earlier papers. Banzhaf: Collected Studies, Research Laboratory Dept. of Health, New York, 1908, 4, p. 230. Homer: J. Hyg., 1916, 15, p. 388. Heinemann: J. Infect. Dis., 1916, 19, p. 433. Besredka: Ann. de l'Inst. Pasteur, 1923, 37, p. 935. Ruppel: Ber. d. deutsch. pharm. Gesellsch., 1920, 30, p. 314.

[2] Rakuzin: Ztschr. f. Immunitätsforsch. v. exper. Therap., 1924, 39, p. 193.

[3] Eli Lilly and Co.: Personal communication, 1925.

[4] Ramon: Compt. rend. Soc. de biol., 1923, 88, p. 167; Compt. rend. Acad. d. Sc., 1923, 176, p. 267.

[5] Ottenberg and Stenbuck: Proc. Soc. Exper. Biol. & Med., 1923, 21, pp. 14, 303.

[6] Huntoon: Personal communication, 1926. Dr. Huntoon has indirect evidence of a concentration exceeding this value by ten times.

[7] Locke; Main and Hirsch: J. Infect. Dis., 1926, 39, p. 126.

titer serums, in accomplishing a low order of concentration of titer, and in eliminating the anaphylactogenic properties of the euglobulin and the readily denatured pseudoglobulin fractions of the serum.

The specific fractionation of diphtheria antitoxin by means of adsorption upon diphtheria toxin was not attempted until 1923 when Ramon [4] first succeeded in producing a flocculation of toxin-antitoxin from their mixture.[8]

Five hundred c c. of fresh diphtheria toxin were added to 40 c c. of antitoxin, containing 200 units per c c. The resulting precipitate of toxin-antitoxin floccules was separated by centrifugation and thoroughly washed, first with physiologic salt solution and finally with distilled water. This preparation was dissolved in 75 c c. of N/100 acetic acid, and heated for one hour at 58-60 C. It was estimated to contain a concentration of 60,000 antitoxic units per gram of the dried floccules, and to represent a recovery of 75% of the original titer.

Composition of the Toxin-Antitoxin Flocculcs.—The precise composition of the floccules separating from neutral mixtures of toxin and antitoxin is not entirely known. They do, of course, contain the toxin [9] and antitoxin [4] in somewhat the same proportion as in the original mixtures, but associated with varying amounts of protein, lipin, and even inorganic salts, depending upon the nature of the components originally in the mixture and upon the manner of preparation.

Flössner and Kutscher [10] report that the Ramon floccules consist of 86.36% magnesium ammonium phosphate, 10% alcohol-insoluble protein, 1.1% alcohol-soluble protein, and 2.52% cholesterol and lipin. We have found that this proportion of inorganic matter will separate if the mixtures are held a long time in the ice-box. It does not separate when flocculation is carried out rapidly at 50 C. and seems to be the result of a secondary precipitation. When correction is made for this inclusion, Flössner and Kutscher's figures become: alcohol-insoluble protein 73.4%, alcohol-soluble protein 8.1%, and lipin 18.5%. These ratios are not unusual for substances of protoplasmic origin.

The statement of Niederhoff [11] that the floccules consist chiefly of lipins is based upon an examination of a precipitate obtained according to the method of Georgi [12] by the addition of a dispersion of cholesterolized beef-heart "antigen" to neutral toxin-antitoxin mixtures. Since his original mixtures contained only a few units of antitoxin, the protein concentration of the separated floccules had of necessity to be exceedingly small—but hardly negligible.

[8] Ramon: Compt. rend. Soc. de biol., 1922, 86, pp. 661, 813; Ann. de l'Inst. Pasteur. 1923, 37, p. 1001.
[9] Glenny, Pope; Waddington and Wallace: J. Path. & Bact., 1925, 28, p. 463.
[10] Flössner and Kutscher: Münch. med. Wchnschr., 1924, 71, p. 576.
[11] Niederhoff, P.: Münch. med. Wchnschr., 1921, 68, p. 330.
[12] Georgi, W.: M. Klink, 1920, 16, p. 1053.

Many antitoxins give a spurious, nonspecific flocculation with toxins [13] which may, perhaps, be attributed to an agglutination of bacillary lipoprotein from the toxin broth by agglutinins in the antitoxin serum. It has been suggested [14] that the floccules consist chiefly of this agglutination precipitate and merely carry down the toxin-antitoxin complex by adsorption.

Hartley has suggested [15] that the toxin-antitoxin complex is adsorbed upon lipins which become flocculated from the serum during the reaction. Friedberger and Ikeda [16] infer that the toxin-antitoxin complex itself is largely a lipoidal aggregate.

The floccules may also contain some coadsorption of nonspecific proteins, of complement, and of cell ferments.

Fractionation of Toxin-Antitoxin Floccules.—The earliest reported fractionation of a specific antigen-antibody precipitate is reported by Landsteiner.[17] His apparent dissociation of antibody from its union with antigen by simple extraction with warm physiologic salt solution has been confirmed by a number of investigators. It may be referable to the low avidity with which a portion of the antibody is bound. [The looseness of the antigen-antibody bond, as indicated by this easy dissociation, has been used as an indicator of low avidity by Mueller,[18] as was the correlated low complement-fixing capacity by Hecht-Johansen.[19]] The whole of the adsorbed antibody is not recovered in this way in the absence of secondary changes [7] nor are the extracts of great purity or avidity.

Extraction of specific precipitates with dilute acids and alkalies [20] has been more successful.

The isoelectric range of the toxin-antitoxin floccules is determined by their composition. It is shifted from P_H 4.7 to 7.2 by ether extraction and is considerably broadened when the floccules are suspended in a medium of high salt concentration. A dispersion of the floccules in a slightly acid or alkaline solution presents a solubility equilibrium differing from that of an isoelectric dispersion in that the solubilities of the different constituents are no longer at a minimum, and therefore, com-

[13] Glenny and Wallace: Jour. Path. & Bact., 1925, 28, p. 317.
[14] Moloney and Weld: J. Path. & Bact., 1925, 28, p. 655.
[15] Brit. J. Exper. Path., 1925, 6, p. 180.
[16] Klin. Wchnschr., 1925, 4, p. 2149.
[17] Münch. med. Wchnschr., 1902, 49, p. 1905.
[18] Kraus and Levaditi: Handbuch der Immunitätsforsch., Supplement 1, 1911.
[19] The Typhoid-Paratyphoid Group of Bacteria and About Avidity, 1923.
[20] Liebermann and Fennyvessy: Centralbl. f. Bakteriol., 1908, 47, p. 274. Kosaki: J. Immunol., 1918, 3, p. 109 (with survey and discussion of the literature). Huntoon: Ibid., 1921, 6, p. 117. Locke and Hirsch: J. Infect. Dis., 1925, 37, p. 449.

parable value. The more lyophobic constituents tend to remain undissolved and the "extract," in our preparations, contains less nitrogen associated with one antitoxic unit than does the sediment.

Isoelectric fractionation of the specific precipitate is much simplified when the combining power of the bound toxin is first reduced either by differential heat destruction,[4] ether extraction (Locke and Hirsch [20]) or electro-dialysis of an acid solution. These procedures may cause a partial denaturation and deterioration in the avidity of the bound antitoxin, for the recovered preparations are often not of a purity and quality equal to those obtained by simple isoelectric fractionation.

Fractionation has also been somewhat simplified by commencing with specific precipitates of greater purity. This was done first by Gay and Chickering,[21] who used a specifically precipitating extract of antigen for the flocculation, in place of whole antigen.

An analysis of the conditions governing the composition of the hemolysin-erythrocyte specific precipitate [7] has demonstrated that the purity of these precipitates is frequently determined by the avidity of the amboceptor serum used. The purity of the toxin-antitoxin floccules should, by analogy, be determined largely by the avidity of the toxin and antitoxin preparations from which they are obtained. We have had some indication that this relation may be true but sufficiently varied samples of toxin and antitoxin have not been available for a critical test. The purest preparations were secured by the simple isoelectric fractionation of floccules obtained from a toxin retaining 85% of its original avidity. The floccules obtained from the same antitoxin and the same toxin after the avidity of the latter had deteriorated to 50% of its original value yielded preparations less than half as pure.

The fractionation procedures summarized in table 2 are self explanatory. In all, some 25 fractionations of floccule preparations were carried out, using approximately, 50 liters of diphtheria toxin, kindly supplied by the Eli Lilly Company.

The purified preparations have the anaphylactogenic character of horse serum protein and do not withstand boiling. They have an isoelectric point very slightly different from that of the original floccules and probably represent dispersions of the same sort of material as composes the whole floccules. Their avidity and therapeutic effectiveness does not excel that of the original antitoxic plasma from which they are obtained.

[21] Chickering, H. T.: J. Exp. Med., 1915, 22, p. 248. Felton and Bailey: J. Infect. Dis., 1926, 38, p. 145.

A significant recovery of toxin or toxoid from the fractionation residues could not be demonstrated.

Avidity.—Avidity is the attractive force or affinity which exists between antibody and antigen. So defined, it has the dimensions of potential energy and requires to be expressed as the product of a quantity factor (the concentration of effective combining units), and an intensity factor (the effectiveness of those units).

The concentration of effective units in a toxin or antitoxin is not necessarily equivalent to its titer unless this value has been obtained in

TABLE 2

THE COMPARATIVE PURITY OF DIFFERENT PREPARATIONS OF DIPHTHERIA ANTITOXIN OBTAINED BY FRACTIONATION OF THE TOXIN-ANTITOXIN FLOCCULES

Nature of the Preparation	Yield, Total Recovery of Antitoxin, %	Purity, Mg. Nitrogen Associated With One Unit of Antitoxin	% of Original, Maximum Avidity Retained by the Toxin Used for the Flocculation	Comment
Electrodialysis of an acid solution of the floccules: Neutralization				
Supernatant.....	..	0.0013	..	Averages of four series
	50	.....	85	
Residue.....	..	0.0065	..	
Simple isoelectric separation: Neutralization				One series only, of each. The values are conservative but not more than 60% accurate
Supernatants.....	..	0.0005 – 0.0015	85	
		0.002	50	
Heating, at 58-60 C. for 1 hour, a N/100 acetic acid solution of the floccules, according to directions of Ramon [4].....	65	0.0025	75	Averages of three series, each
	45	0.004	50	
Ether extraction of an acid solution of the floccules: Neutralization				One series only, of each. 60% of the recovered antitoxin was concentrated into the better fraction
Supernatant.....	..	0.0020	..	
	65	.....	75	
Residue.....	..	0.0047	..	
Whole preparation..	45	0.004	50	

such a way that the measurements are independent of fluctuation in the intensity factor. The titer of an unmodified antitoxin serum, when determined by subcutaneous injection into guinea-pigs of mixtures with a standard toxin, is not directly affected by the changes in avidity caused by aging or moderate heating. It may be regarded as, approximately, a quantity value.

The L + value of a toxin is, on the other hand, an avidity value. It represents the toxicity or capacity of the toxin to unite with certain tissues of the host in lethal concentration. This toxic property may

become decreased with aging, moderate heating, formolization, etc., with little corresponding decrease in the antitoxin-binding or antigenic titer, as measured by the Lf value,* and is modified by the size and character of the animal into which it is injected.

A quantitative measure of the avidity of a diphtheria antitoxin preparation requires the determination of distributions which are not, at this time, feasible. However, there is a close and obvious correlation between the avidity of an antitoxin and the product of its titer by a function of the time required for its "complete" combination with a given toxin.

The combination time is measured as a function of the time which must elapse before a mixture of toxin and antitoxin, containing the latter in slight excess, may be injected intravenously into young rabbits without lethal consequence. It may be seen, from table 3, to be somewhat increased by aging and heating, permitting the conclusion that the avidity of an antitoxin is injured by these processes. The decreased avidity, per unit, of commercially concentrated antitoxin may be due, in part, to the prolonged heatings during their preparation. But since these heatings also decrease the anaphylactogenic capacity of the associated serum proteins, they are not undesirable.

A simple, indirect measure of the combination time was suggested by Ramon [8] in 1923. He observed differences in the flocculation times of different antitoxin preparations of identical titer and stated that these differences might be due to differences in avidity. This suggested relation has since been confirmed by Madsen and Schmidt,[22] and, independently, by ourselves. The flocculation time is an excellent index of the combination time of an unmodified diphtheria antitoxin serum, but it is in no sense a quantitative measure nor can it be recommended for the examination of purified preparations.

Graded quantities of the antitoxin preparation are measured into the bottoms of a series of micro test tubes, 2 cc. of toxin are added, and the tubes gently shaken. They become somewhat opalescent upon continued standing at room temperature and are then centrifugated. The tube in which a coherent sediment is first produced is taken as the indicating time and Lf value tube. The sediments are readily visible when caused to swirl slightly under strong illumination. Concentrated preparations may be titrated directly by this modified method.

Therapeutic Usefulness.—Diphtheria antitoxin is administered for the purpose of checking further adsorption of toxin by the susceptible

* Ehrlich's "spectrum" of prototoxin, toxin, toxoid, toxone, etc., becomes interpreted, upon the avidity hypothesis, as an application of the law of probability to the distribution of energy (avidity) among the individual antigenic aggregates of a toxin preparation. It is possible that this application may also underlie differentiations in microbic virulence (Ref. 9, p. 279).

[22] Ann. de l'Inst. Pasteur, 1926, 40, p. 300.

tissues of the host. It is therapeutically most useful if it performs this function rapidly, that its administration may not be to late; completely, that postdiphtheritic manifestations may not occur, and, specifically, without the accompaniment of serum sickness. The first two conditions require that the antitoxin be sufficiently avid; the last that it be purified as much as possible from associated proteins.

A desired avidity (total unitage times unit effectiveness) may be obtained with any serum, no matter how poor, providing that it be injected in sufficiently large and rapidly absorbed dosage. The threshold avidity which an antitoxin must have to be therapeutically useful * is determined by the size and susceptibility of the host and by the total

TABLE 3

THE AVIDITY AND THERAPEUTIC USEFULNESS OF DIFFERENT TYPES OF DIPHTHERIA ANTITOXIN AS INDICATED BY THEIR CAPACITY TO NEUTRALIZE A MATURE TOXIN

Nature of Antitoxin Preparation	Cc. of Antitoxin Required to Neutralize the Simultaneous Injection of 1 Lo Unit of Mature Toxin Into			Flocculation Time of the Lf Mixture, Hours	Combination Time of the Lo Mixture, Hours
	Guinea-Pigs		Rabbits,		
	Subcutaneous	Intracardial	Intravenous		
Fresh plasma *	0.0008	.....	0.0008	0.5	0.25
Fresh plasma heated at 56 C. 1 hour	0.0008	0.0008	0.0025†	2.5	2.0†
Old plasma	0.0033	0.0037	0.016	7.0	5.0
Pseudoglobulin preparation	0.00070	0.00072	0.0022	20.0	2.5
Floccule preparation	0.04	.....	0.12	...	2.5

* Sample received from Dr. F. M. Huntoon.
† Rabbits showed marked variations in the protection conferred by this preparation.

concentration and rate of additional introduction of the free toxin to be neutralized. The greater susceptibility of the rabbit to the toxin (table 3) requires of an antitoxin a greater threshold avidity than does the lower susceptibility of the guinea-pig. The comparative susceptibility of man to diphtheria toxin could not be determined.

SUMMARY

Highly purified preparations of diphtheria antitoxin may be secured by isoelectric fractionation of the Ramon toxin-antitoxin precipitate. These preparations contain from 0.0005 to 0.0015 mg. of nitrogen per antitoxic unit; have the anaphylactogenic character of horse serum protein; and do not withstand boiling. The avidity and therapeutic usefulness of antitoxin preparations are diminished by aging and heating.

* The early work upon a possible relation between the avidity of an antitoxin and its therapeutic usefulness is well summarized and discussed by Mueller.[18] The early conception of avidity differed from that proposed in this paper. It was thought to be a separate property, independent of the titer of the serum and corresponding, roughly, to the "intensity factor" already discussed.

DISTRIBUTION OF THE SPORES OF BACILLUS BOTULINUS AND BACILLUS TETANI IN THE SOIL

1. IN MARYLAND

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Studies by a number of investigators have shown that B. botulinus and B. tetani are, in general, apparently rather widespread in nature, and that they occur with considerable frequency in the soil in certain localities. Thus Meyer and Dubovsky [1] and Dubovsky and Meyer [2] have carried out examinations of soil samples obtained from almost all parts of the United States, and have shown that as high as 27% of such specimens yielded toxic substances in cultures, as shown by the intoxication with typical symptoms in animals given injections of the filtrates. This study, in which 2,162 soil specimens were examined, is by far the most extensive general survey that has ever been made, but there are also other records in which the frequency of occurrence of B. botulinus and B. tetani has been determined within a limited area, such as a certain county or state. Thus Burke,[3] Meyer and Dubovsky,[4] and Hall and Peterson [5] have studied the distribution of these organisms in the soil of California particularly, while Tanner and Dack [6] have carried out similar investigations of the soil of Illinois.

It was because of the availability of these data for comparison, and because of the general lack of information in regard to the distribution of these organisms in the soil of the states along the Atlantic Seaboard, that the present study was undertaken. In this report we present the results of an intensive survey of the soil of Maryland with regard to the occurrence of toxic anaerobes—specifically B. botulinus and B. tetani—and in subsequent communications it is hoped that further data in regard to their occurrence in other states bordering on the Atlantic, may be presented.

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[1] J. Infect. Dis., 1922, 31, p. 559.
[2] Ibid., p. 614.
[3] J. Bact., 1919, 4. p. 541.
[4] J. Infect. Dis., 1922, 31, p. 541.
[5] J. Bact., 1924, 9, p. 201.
[6] J. Infect. Dis., 1922, 31, p. 92.

So far as we have been able to learn from the literature, the only examinations of Maryland soil, with the above mentioned objective in mind, that have been reported heretofore, are those of Geiger, Dickson, and Meyer,[7] Bengtson,[8] and Meyer and Dubovsky.[1] The numbers of soil samples examined and the results obtained by these workers are presented in table 1.

The tabulated results show that B. botulinus, in its various types, was present in Maryland soil in a relatively high percentage of the samples examined. This fact is further emphasized when the frequency of occurrence of these organisms in the soil of Maryland is compared with the frequency of their occurrence in the soil of other states, as determined by Meyer and Dubovsky,[1] as it is found that soil samples from Kentucky and Oregon alone yielded a greater percentage of toxic cultures. In other words, so far as we know, toxic organisms are more common in Maryland soil than in that of any other state with the exceptions noted.

TABLE 1

RESULTS OF PREVIOUS STUDIES OF THE SOIL OF MARYLAND

Investigator	Soil Specimens Examined, Number	Toxic Cultures		Identification of Toxin	
		Number	%	Number	Type
Meyer and Dubovsky	22	17	77.3	17	B. botulinus, types A and B
Geiger	Not stated	Not stated	54.0	Not stated	Not stated
Bengtson	1	1	100.0	1	B. botulinus, type C
Average			77.1		

In order to make our study comparable with that of other workers, our soil samples were taken from areas similar to those they investigated: from virgin soil, garden patch, corn field, pea field and tomato field. At the same time the technic of culturing from the samples and the method of testing the cultures for toxicity, followed the general scheme employed by the earlier workers, with certain variations as indicated below under the discussion of the technic employed.

EXPERIMENTAL WORK

Sources of Soil Specimens.—In this study 62 samples of soil were collected in sterile containers from 10 of the 23 counties of Maryland: from Dorchester, 5 specimens; Carroll, 5; Wicomico, 5; Charles, 5;

[7] U. S. P. H. S., Public Health Bull. 127, 1922, p. 56.
[8] Hygienic Lab. Bull. 136, 1924, p. 38.
[9] J. Infect. Dis., 1922, 31, p. 501.

Frederick, 9; Allegany, 6; Worcester, 5; Cecil, 5; Baltimore, 12, and Ann Arundel, 5. They represent quite different types of soils, which may be characterized as clay, humus, sandy loam, and gravelly loam.

The accompanying map of the state of Maryland shows that the samples came from all different parts of the state; from the more rugged and hilly area of the western part of the state as well as from the level and more highly agricultural section of the Eastern Shore.

Method of Culturing Soil and Identifying Toxin.—The technic employed in culturing all the samples was the same.

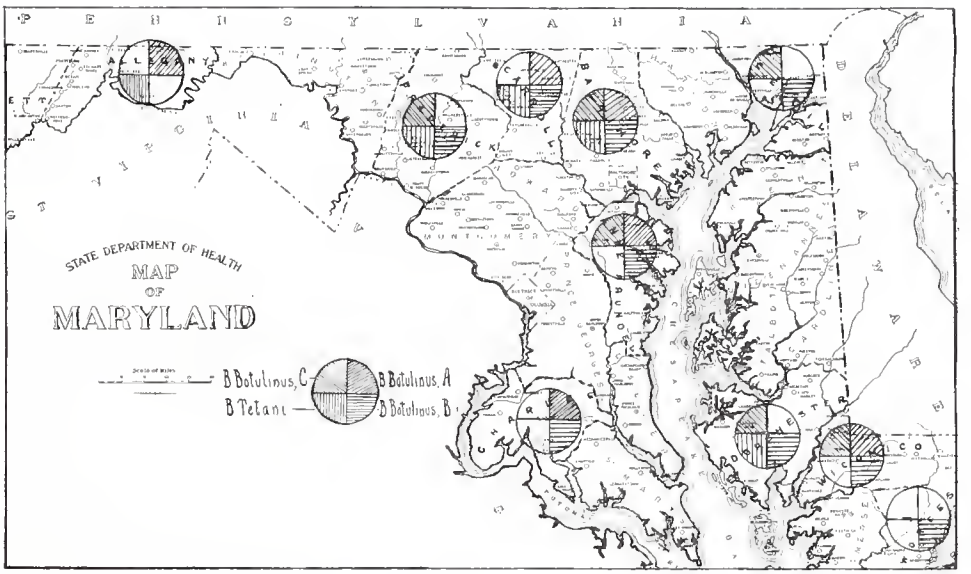

Fig. 1.—Sources of soil samples, and the types of toxic organisms found in the cultures.

When each container was opened for analysis, 30 Gm. of soil were weighed out under conditions that precluded contamination. This amount of the sample was then thoroughly mixed with about 30 cc. of sterile salt solution in a flask and heated in a water bath for one hour at 80 C. In this way nonsporulating contaminants were eliminated and the spore forms of aerobes and anaerobes alone remained.

After the period of heating, the suspension of soil was poured into a flask containing 100 cc. of the freshly boiled beef heart peptic digest broth, recommended by Meyer and Dubovsky[9] for use in soil examination. After thoroughly mixing the soil suspension and the medium, melted vaseline was poured over the culture to a depth of about 0.5 cm. and the flask was then incubated at 37.5 C. for 10 days to allow the formation of toxin in the culture. Fifteen to 20 cc. of the supernatant fluid were then removed from the culture flask by means of a sterile pipet, placed in a sterile centrifuge tube, and centrifugalized for 30 minutes to throw down as much as possible of the suspended material. The upper clear portion was then filtered through a Mandler diatomaceous filter and the sterility tested by culturing

1 to 2 cc. anaerobically in chopped meat. In the meantime the remainder of the filtrate was kept at a low temperature in the icebox until the results of the sterility test could be determined, after which it was used for animal inoculation.

In order to identify the type of toxin present in the filtrates, inoculation tests were made with each filtrate in 4 guinea-pigs. Three were given protective doses of botulinus antitoxin and the fourth received a protective dose of tetanus antitoxin, using the same amount of antitoxin (0.5 cc.) intraperitoneally in each pig, which later received intraperitoneally 2 cc. of the culture filtrate.

These animals were kept under observation for the development of symptoms, and necropsies were made on the animals that died.

In the interpretation of the results, it becomes clear that the death of any three of the animals and the survival of the fourth will presumably establish the fact that the specific type of antitoxin given that pig protected it against the toxin present in the filtrate. This tentative conclusion supported by observing the development of the typical symptoms of intoxication caused by the type of organism involved, was further confirmed by the isolation in pure culture of this organism in a certain number of instances, as indicated in table 2. A tabular illustration of the method and results in a single series of inoculations follows.

PROTOCOL OF TOXIN IDENTIFICATION TESTS

Guinea-pig		Antitoxin Given, 0.5 cc.					
Number	Weight, Gm.	Type	Units		Symptoms	Death (in Days)	Conclusion: Type of Toxin in Filtrate
1	420	Botulinus, A	700	Each guinea-pig given 2 cc. culture filtrate	Botulism	2	B. botulinus, type B
2	400	Botulinus, B	50		None	Survived	
3	395	Botulinus, C	200		Botulism	2	
4	480	Tetanus	150		Botulism	2	

A toxic substance of considerable potency must have been present in the culture filtrate, for three of the four pigs died within two days with typical symptoms of botulism, whereas the fourth pig was able to survive indefinitely. This would indicate that the botulinus antitoxin given the surviving guinea-pig neutralized the toxin of the culture filtrate, and justifies the tentative conclusion that this toxin is identical with that produced by B. botulinus, type B. In this manner the 62 spoil specimens were examined for the presence of toxin producing organisms (table 2).

A summary of the detailed results of the examination of soil culture filtrates is given in table 3.

From the tabulation it will be seen that of the total number of 62 soil culture filtrates examined, 5 were found to be entirely nontoxic, as indicated by animal inoculation. Further, 4 culture filtrates were weakly toxic, as indicated by the development of symptoms of intoxication in the guinea-pigs, but could not be typed by the method employed. Of the remaining culture filtrates examined, 37 yielded weakly toxic substances which were of sufficient potency to enable typing, while 20

TABLE 2

DETAILED RESULTS OF THE EXAMINATIONS OF CULTURES OF 62 SOIL SPECIMENS

Soil Sample Number	Sources of Samples (County)	Type of Soil	Crop Grown on Soil	Symptoms in Guinea-Pigs Given 2 cc. Filtrate of 10 Day Culture	Type Antitoxin Given Surviving Guinea-Pig: Indicating Apparent Type Toxin in Filtrate	Toxin Quality	Organism Isolated
1	Dorchester	Humus	None (virgin)	Botulism	C		B. botulinus, C
2	Dorchester	Sandyloam	Pea	Botulism	B		.....
3	Baltimore	Sandyloam	Pea	Botulism	B		B. botulinus, B
4	Baltimore	Sandyloam	Corn	Botulism	Botulinus A		B. botulinus, A
5	Baltimroe	Clay	Tomato	Botulism	C		B. botulinus, C
6	Baltimore	Humus	None (virgin)	Botulism	B		.....
7	Charles	Sandyloam	Vegetable	Botulism	B		B. botulinus, B
8	Charles	Sandyloam	Pea	Botulism	B		.....
9	Allegany	Clay	Tomato	Tetanus		Cul-	.....
9	Allegany	Clay	Tomato	Tetanus	Tetanus	Cul-	.....
10	Allegany	Clay	Pea	Botulism	B	tures	.....
11	Allegany	Clay	Corn	Botulism	A	1 to 20,	B. botulinus, A
12	Worcester	Sandy	Corn	Botulism	B	strong	.....
13	Worcester	Sandy	Vegetable	Botulism	B		.....
14	Worcester	Sandy	None (virgin)	Botulism	B		.....
15	Worcester	Sandy	Tomato	Botulism	Botulinus B		B. botulinus, B
16	Cecil	Sandy	Corn	Botulism	B		.....
17	Cecil	Sandy	None (virgin)	Botulism	B		.....
18	Frederick	Sandy	Vegetable	Botulism	B		.....
19	Ann Arundel	Sandy	Pea	Botulism	A,C		.....
20	Ann Arundel	Sandy	Corn	Botulism	B,C		.....
21	Dorchester	Clay	Vegetable	Botulism	B		.....
22	Dorchester	Clay	Tomato	Botulism	C		.....
23	Dorchester	Sandyloam	Corn	Tetanus	Tetanus		B. tetani
24	Baltimore	Sandyloam	Vegetable	Tetanus	Tetanus		.....
25	Baltimore	Clay	None (virgin)	Botulism	Botulinus,B		.....
26	Carroll	Clay	Pea	Tetanus	Tetanus		.....
27	Carroll	Clay	Corn	Botulism	A		.....
28	Carroll	Clay	Tomato	Botulism	A		.....
29	Carroll	Humus	None (virgin)	Botulism	A		.....
30	Carroll	Clay	Vegetable	Botulism	B		B. botulinus, B
31	Wicomico	Sandy	Vegetable	Botulism	A,C		.....
32	Wicomico	Humus	None (virgin)	Botulism	Botulinus B		.....
33	Wicomico	Sandy	Corn	Botulism	B		.....
34	Wicomico	Sandy	Tomato	Botulism	B		.....
35	Wicomico	Sandy	Pea	Botulism	A	Cul-	B. botulinus, A
36	Charles	Sandy	Corn	Botulism	A	tures	.....
37	Charles	Humus	None (virgin)	Botulism	A	21 to 53	.....
38	Charles	Sandy	Tomato	Botulism	B	weak	.....
39	Frederick	Clay	Pea	Tetanus	Tetanus		.....
40	Frederick	Clay	Tomato	Botulism	Botulinus,B		.....
41	Frederick	Sandy	Corn	Botulism	B		.....
42	Frederick	Humus	None (virgin)	Botulism	Botulinus C		.....
43	Frederick	Sandy	Vegetable	Botulism	C		B. botulinus, C
44	Allegany	Humus	None (virgin)	Tetanus	Tetanus		.....
45	Worcester	Sandy	Pea	Botulism	B		.....
46	Cecil	Clay	Tomato	Botulism	A,C		.....
47	Cecil	Humus	None (virgin)	Botulism	B		.....
48	Cecil	Humus	None (virgin)	Botulism	B		.....
49	Baltimore	Sandy	Tomato	Botulism	Botulinus A,C		.....
50	Baltimore	Sandy	Corn	Botulism	B,C		.....
51	Frederick	Sandy	Tomato	Botulism	B,C		.....
52	Ann Arundel	Sandy	Vegetable	Botulism	B,C		.....
53	Ann Arundel	Sandy	None (virgin)	Botulism	B,C		.....
54	Allegany	Sandy	Vegetable	Botulism	A,B,C	Toxin	.....
55	Baltimore	Sandy	Garden veg.	Botulism	Botulinus A,B,C	un-	.....
56	Baltimore	Clay	Tomato	Botulism	A,B,C	typed	.....
57	Baltimore	Clay	Potato	Botulism	A,B,C		.....
58	Baltimore	Humus	None (virgin)	No symptom	Botulinus		.....
59	Frederick	Gravelly loam	Pea	No symptom	A,B,C,		.....
60	Frederick	Sandy	Corn	No symptom	and		.....
61	Ann Arundel	Sandy	Tomato	No symptom	tetanus		.....
62	Allegany	Clay	None (virgin)	No symptom	(nontoxic)		.....

may be classified as strongly toxic and presented no difficulties in the determination of type.

The percentage frequency of the occurrence of the different types of toxin in the culture filtrates is indicated in table 4.

TABLE 3

SUMMARY OF TESTS FOR TOXICITY OF SOIL CULTURE FILTRATES

Soil Samples	County	Specimens Examined	Strongly Toxic Cultures	Weakly Toxic Cultures	Untyped Weakly Toxic Cultures	Nontoxic Cultures
1	Dorchester	5	2	3	0	0
2	Carroll	5	0	5	0	0
3	Wicomico	5	0	5	0	0
4	Charles	5	2	3	0	0
5	Frederick	9	1	6	0	2
6	Allegany	6	3	2	1	1
7	Worcester	5	4	1	0	0
8	Cecil	5	2	3	0	0
9	Baltimore	12	4	7	3	1
10	Ann Arundel	5	2	2	0	1
	Total	62	20	37	4 Includes untyped weakly toxic	5

TABLE 4

FREQUENCY OCCURRENCE OF THE DIFFERENT TYPES OF TOXIN

	Kind of Toxin	Number Containing Toxin	Percentage Frequency
In the 37 Weakly Toxic Filtrates	Botulinus		
	Type A	6	16.2
	Type B	12	32.4
	Type C	3	8.1
	Types A and C	3	8.1
	Types B and C	4	10.9
	Tetanus	5	13.5
	Untyped	4	10.9
In the 20 Strongly Toxic Filtrates	Botulinus		
	Type A	2	10
	Type B	3	15
	Type C	2	10
	Types A and C	1	5
	Types B and C	1	5
	Tetanus	1	5

RESULTS AND DISCUSSION

In this investigation we have examined soil specimens from virgin soil, tomato fields, pea fields, corn fields, and garden patches; sources that would enable us to compare our results with those obtained by others, such as Meyer and Dubovsky, and Hall and Peterson in California, and Tanner and Dack in Illinois.

Comparing the results of our examination of 12 samples of virgin soil, in which 11 of the samples yielded toxic culture filtrates, with those of Meyer and

Dubovsky, we find that these investigators obtained 45 toxic cultures from 78 samples of similar material. This gives a percentage incidence of 91.7 in the virgin soil of Maryland as compared with 57.6 in California soil.

On the other hand, the examination of 50 samples of Maryland garden and cultivated field soils yielded 46 toxic culture filtrates, whereas Meyer and Dubovsky obtained only 59 toxic cultures in the course of the analysis of 226 samples; while Hall and Peterson obtained 14 toxic filtrates from the 20 specimens of California soil examined by them, and Tanner and Dack demonstrated toxic substances in 6 of 31 specimens from Illinois. In other words, we found 92.0% of the Maryland cultivated field soil contained toxin-producing organisms, as compared with the 26.9% obtained by Meyer and Dubovsky and the 70% obtained by Hall and Peterson in their examinations of California soil, and 19.3% obtained by Tanner and Dack in Illinois.

Summarizing the results obtained by the various investigators in California and Illinois, in comparison with our results in Maryland, we find that in a total of 304 specimens of virgin and cultivated field soil examined in California by Meyer and Dubovsky, 104 samples (34.2%) were shown to contain toxic substances when cultured; and Hall and Peterson demonstrated the presence of toxins in 14 samples (70%) of soil in California examined by them; while Tanner and Dack showed 8 cultures (24.2%) made from samples of Illinois soil,

	Botulinus			
	Type A	Type B	Type C	Tetanus
California				
Meyer and Dubovsky	63.4	6.7	...	....
Hall and Peterson	50.0	15.0	...	20.0
Illinois				
Tanner and Dack	....	15.2	...	....
Maryland	14.0	43.8	8.7	10.5

were toxic. As compared with these results we found 57 of the 62 (91.9%) Maryland soil sample cultures to contain toxins.

In regard to the proportion of the types of B. botulinus identified, by antitoxin protection tests, as present in the total number of soil samples examined, we find that type A was present in 66 (63.4%) of the 104 toxic samples of California soil, as determined by Meyer and Dubovsky, while Hall and Peterson demonstrated it in 7 (50%) of their filtrates of similar material, whereas we found it present in 8 (14%) of 57 toxic samples of Maryland soil. On the other hand, it was found that type B was present alone in 7 (6.7%) and in 2 (15%) of the toxic samples in California, according to Meyer and Dubovsky and Hall and Peterson respectively, while Tanner and Dack found it in 5 (15.2%) of their Illinois samples, and we demonstrated it in 25 (43.8%) of the 57 samples of Maryland soil. In addition, we found type C present in 5 (8.7%) of the 57 toxic samples of Maryland soil. The determination of this type in their samples was not made by any of the other investigators. Finally, we identified B. tetani in 6 (10.5%) of our 57 toxic cultures, whereas Hall and Peterson showed it to be present in 20% of their samples of California soil. A summary of the frequency of identification (% of samples tested) of the different types of toxins may be given.

From this survey of the various studies that have been made concerning the frequency of occurrence of B. tetani and the different types

of B. botulinus, it becomes clear—as has been pointed out previously by Meyer and Dubovsky and by Hall and Peterson—that B. botulinus, type A, occurs much more frequently in California soil than in that from the eastern part of the United States. In fact, type A occurs about four times as often in California as in Maryland. On the other hand, type B predominates in Maryland soil in approximately the same proportion. Further, it is to be noted that type C is rather frequently found in Maryland soil, although there are no findings in the California

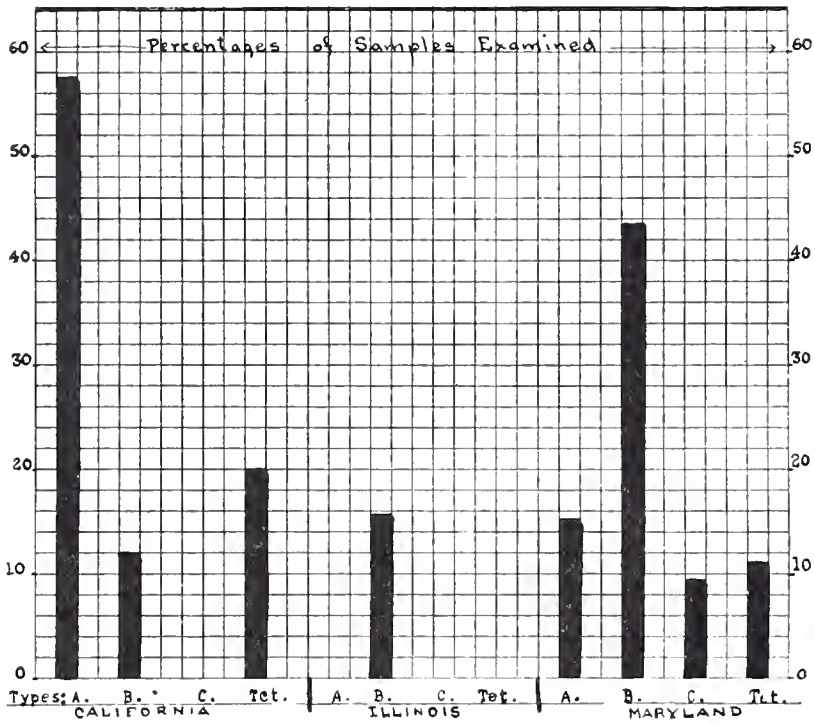

Fig. 2.—Frequency of identification (% of samples) of toxic anaerobes in soils of California, Illinois and Maryland (B. botulinus types A, B, and C, and B. tetani [Tet.])

and Illinois studies with which to make a comparison, as this type was not sought for by the investigators cited above. Finally, it will be seen that B. tetani occurs almost twice as frequently in the California studies as in ours in Maryland. The comparative results obtained in the California, Illinois and Maryland investigations are indicated in figure 2.

Thus far we have discussed our results in relation to those obtained by other workers in which a single type of toxic organism alone was demonstrated in the soil sample cultures. It should be mentioned, however, that in all the soil surveys reported there have been certain

instances in which two types of toxins were demonstrated in a single culture. Thus we find that B. botulinus, types A and B, were shown by Meyer and Dubovsky to be present together in 3 out of 78 samples of virgin soil and in 1 of 226 samples of garden and field soil; while Hall and Peterson found these two types present together in 2 of the 20 soil cultures they examined in California. In our study of Maryland soil we failed to find these two types present in any single sample, although types A and C and B and C were present, respectively, in 4 and 5 of the 62 samples. On the other hand, Hall and Peterson found B. botulinus and B. tetani present together in one of their samples, whereas we failed to demonstrate this combination as present in any of our culture filtrates.

Up to this point in the discussion as to the prevalence of toxic substances in soil culture filtrates, we have compared our results with those of other investigators, using for comparison the total numbers of toxin containing cultures in each case. That is, we have not differentiated between those cultures which might be regarded as strongly toxic and those which might be designated as weakly toxic. If we may now define these terms, it may be said that we have arbitrarily called any culture filtrate strongly toxic if, when injected in the amount of 2 cc., it caused the death of the animal acutely, with characteristic symptoms of botulism or tetanus within 3 days. On the other hand, we believe that a culture filtrate may be considered as containing a weak toxin if, injected in the amount stated, it causes the animals to become chronically ill with typical symptoms and eventually kills them in from 4 to 8 days.

On the basis of these definitions, it may be said that in this investigation we have found 20 of the 57 toxin containing filtrates to belong to the class of strong toxins. The remaining 37 toxic filtrates contained weak toxins. The classification of the type of toxin found in each toxic soil sample is indicated in table 2.

In any such study as this the determination as to whether one or another toxic organism is present in any individual soil may be tentatively established on the basis of antitoxin protection tests following the technique indicated in the illustration of the results obtained in toxin identification, given previously in this paper. It should be realized, however, that the establishment of the presence of any particular type of toxin in a sample by this method should be regarded as only tentative until its presence is confirmed by its actual isolation in pure culture and identification assured on the basis of cultural and toxicological tests. We considered it necessary, therefore, to confirm by isolation the tenta-

tive identification of the various types of toxic organisms indicated as present in a certain number of our soil samples, and several cultures from both the strongly toxic and weakly toxic group were selected and isolation attempted, with the results indicated in table 2. From the results it will be seen that in every one of these unselected samples isolation confirmed the tentative conclusion as to the type of toxic organism present on the basis of antitoxin protection tests, and it would not seem unreasonable to believe that further confirmation might have been obtained in the case of any individual sample had it been desired.

In conclusion, it may be said that in this investigation no cultivations of soil samples were made in flasks that had been previously used for similar examinations. In this way we obviated the difficulty encountered by Meyer and Dubovsky in their work, in which it was shown that the spores of toxin producing organisms might survive the cleaning and sterilizing process to which the glassware was subjected, and subsequently lead to the production of toxin in these flasks when they were again used.

SUMMARY

In this investigation 62 soil samples, obtained from various localities throughout the state of Maryland, have been examined for their content of toxin producing anaerobes. Five of these samples were found to contain no such organisms. Four of the remaining samples contained toxin producing organisms that could not be identified by the methods employed, that is, the toxins produced were not neutralized in the animal body by the injection of tetanus antitoxin or by the antitoxin of the various types of B. botulinus. Of the remaining 53 toxin containing cultures 33 were shown to be relatively weak, but nevertheless the toxins in the cultures were sufficiently potent to be identified by toxin-antitoxin protection tests. The other 20 toxin containing cultures were arbitrarily classified as strongly toxic and were identified by the same method as those above.

In each of the toxic groups in which the presence of specific organisms was tentatively determined by antitoxin protection tests, this deduction was verified by isolating in pure culture the type of organism presumed to be present. These cultures were then identified culturally and by specific antitoxin protection tests in animals.

A brief outline of the results of the examination of the 62 soil specimens is as follows:

Five contained no toxin producing organisms demonstrable by the methods used.

Four contained toxin producing organisms that could not be identified by our antitoxin protection tests.

Bacillus tetani was present alone in 6 samples.

Bacillus botulinus, type A, was present alone in 8; Bacillus botulinus, type B, was present alone in 25; and Bacillus botulinus, type C, was present alone in 5 samples.

Bacillus botulinus, types A and C, were present together in 4, and Bacillus botulinus, types B and C, were present together in 5 samples.

The three types of B. botulinus were not found together, and neither were Bacillus botulinus and Bacillus tetani found together in any single sample.

The sources of the soil samples, and a comparison of our results with those of similar surveys by workers in California and Illinois are shown in illustrations.

EFFECT OF THE CALCIUM, VITAMIN C, VITAMIN D RATIO IN DIET ON THE PERMEABILITY OF INTESTINAL WALL TO BACTERIA

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This study on intestinal resistance was planned in an effort to explain why it is that bacilli which are normally found in the intestines sometimes migrate through the intestinal walls and get into the blood stream. In our study of the need of a dietary balance between calcium, vitamin C, vitamin D in maintaining resistance to tuberculosis,[1] we noticed that when we injected small doses of a human strain of the tubercle bacilli subcutaneously into guinea-pigs, and kept them on a semiscorbutic diet, we often found organisms other than the tubercle bacillus in the heart blood. There were white, waxy nodules on the liver and spleen which could easily be mistaken for true tubercles, but most of them were surface nodules, not deep seated, as is the true tubercle. A bacteriologic study of the organism identifies it as belonging to the paratyphoid group. It increases the alkalinity of milk, produces acid on dextrose, is gram-negative, and is an actively motile, and may be called the aertrycke type (B. aertrycke).[2]

As the heart blood of scorbutic guinea-pigs is usually sterile, and as we have never found lesions caused by this aertrycke type organism in uncomplicated scurvy, we connected its presence in the spleens and livers of our guinea-pigs with the tuberculous infection. Our hypothesis is that the presence of the tuberculous infection increased the need for some factor in the diet of our guinea-pigs, until the lesions caused by a deficiency of this factor were made so severe that bacteria which were normal to the intestines could migrate through the intestinal walls and invade the blood stream, lungs, liver and spleen.

This hypothesis was strengthened by our next findings. In this study we were giving a pronounced excess of cod liver oil, together with a total deficiency of vitamin C, in an effort to hasten the onset of scurvy. Mouriquand[3] and Bezssonov[4] find that cod liver oil is very

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[1] Grant, A. H.; Bowen, J. A., and Stegeman, D. E.: Unpublished data.
[2] Jordon, E. O.: J. Infect. Dis., 1923, 33, p. 567.
[3] Compt. rend. Soc. de biol., 1922, 86, p. 1170.
[4] Compt. rend. Acad. d. Sci., 1922, 75, p. 846; Bull. Soc. Hyg. aliment., 1923, 11, p. 14.

toxic when there is a deficiency of vitamin C in the diet. Bezssonov suggests that this toxicity is due to an excess of vitamin D. Both investigators find that the harmful effects of cod liver oil is overcome by the addition of the vitamin C of oranges and lemons to the diet. In our study the excess of vitamin D did not hasten either the onset or the progress of scurvy, but it did exaggerate some of the lesions. The vitamin D fed guinea-pigs died with scurvy at about the same time as the controls. That the excess of vitamin D had seriously exaggerated the deficiency of vitamin C was not evident at first, but was shown at necropsy.

A large proportion of the guinea-pigs were infected with the same bacillus that so often complicates guinea-pig tuberculosis. In a few there were signs of a concurrent pneumonia, but as a rule the lungs were normal except for this specific infection. The heart blood was usually positive for this organism, and at times the spleen and liver showed the characteristic nodules that we found in our tuberculous guinea-pigs on semiscorbutic diets, but the lungs were infected before either the liver or spleen. The lesions in the lungs were characteristic in all cases examined. The apices of the right upper lobes were the first areas to be infected, the lesions rapidly spreading through the entire lobes. The left upper lobe was infected next, first the apices, and then the lower margin. The small lobes on both sides were usually infected whenever there was any sign of infection in the larger lobes. The lower lobes were not infected except in a small minority of the more severe cases, and then only the upper margins showed the characteristic lesions—tiny nodules in a consolidated area. The consolidations were sharply circumscribed, and resembled the hepatization of pneumonia, except for the characteristic mottled appearance, caused by the tiny nodules. Like the lesions of lobar pneumonia, this infection passes through the stages of red and gray hepatization. After the onset of the gray hepatization the infected areas become very solid and nodular.

As these guinea-pigs were on diets deficient in vitamin C, and as we have found that an excess of vitamin D is most harmful when there is a deficiency of vitamin C in the diet, we sought for an explanation of the marked decrease in the resistance of the intestinal walls in an exaggeration of the lesions of scurvy.

Hojer and Wesin,[5] and Wolbach and Howe,[6] in extended histologic studies of scorbutic guinea-pigs, conclude that the essential lesion in scurvy is a failure of the highly specialized cells of the body to secrete substances necessary for the maintenance of normal tissue. Hojer emphasizes the changes in the secretion of odontoblasts, osteoblasts, and fibroblasts; while Wolbach and Howe extend their studies to include the secretion of all intercellular substances. They conclude that the scorbutic state may be characterized as one affecting supporting tissues in which the cells are unable to produce and maintain intercellular substances: They find that this applies to connective tissue as well as to the matrix of bone and the dentin of teeth, and by inference, to other intercellular substances, including that of blood vessels. Findlay[7] and Jackson and Moore[8] also find that there are marked changes in the intercellular substances in scurvy. Findlay concludes that the essential disturbance in scurvy is an interference with the nutrition of the capillary endothelium, resulting in the stagnation of the blood in the capillaries. He considers the hemorrhages as secondary to the wearing away of the intercellular

[5] Dental Cosmos, 1925, 67, p. 1.
[6] Prac. Soc. Exper. Biol. & Med., 1925, p. 400; Arch. Path. & Exper. Med., 1926, 1, p. 1.
[7] J. Path. & Bact., 1921, 24, p. 446.
[8] J. Infect. Dis., 1916, 19, p. 478.

substance of the capillary walls. Jackson finds that the walls of veins in the vicinity of hemorrhages are sometimes thinned in certain places, with separation of the parts by red corpuscles, and occasionally by a complete break. Although Findlay could not find any instances of a complete break in the vessel walls, he found many places which strongly suggested that red corpuscles were escaping by diapedesis through the weakened intercellular cement substance of the capillary endothelium. McCarrison[9] finds that there are pronounced changes in the intestinal walls in experimental scurvy. In guinea-pigs fed a scorbutic diet of crushed oats and autoclaved milk, he found that the intestines were usually congested, and that there were often atrophic and necrotic changes in the cellular elements of the mucous membrane.

In our studies on the need of a balance between calcium, vitamin C and vitamin D, we find an abundance of evidence that an excess of vitamin D exaggerates many of the lesions of scurvy. We also find that a prolonged excess of vitamin C, together with a total deficiency of vitamin D, often results in somewhat the same defects as an excess of vitamin D, with a deficiency of vitamin C, though the lesions are not so pronounced. In the study of ulcers,[10] we find that when an excess of vitamin D (cod liver oil) is given with a scorbutic diet there is a complete failure of epithelium and the underlying mucosa to regenerate after a severe injury. Severe ulcers (experimentally produced) enlarge and become filled with necrotic pus, the walls thicken, and become purplish within a much shorter time than when the scorbutic diet is given without the excess of vitamin D; while large, pus filled ulcers heal quickly and completely when there is an abundance of both vitamin C and vitamin D in the diet. There is delayed healing when there has been a prolonged excess of vitamin C, with a total deficiency of vitamin D, before the formation of the ulcer. Under these conditions the ulcers do not become larger. The necrotic material formed in the production of the ulcer dries, but although the ulcer does not enlarge, there is a complete absence of any signs of healing. Other studies,[1, 11] including those on the nutritive requirements of nursing mothers, reveal the need for an adequate balance between vitamin C and vitamin D in the diet in order to maintain the normal tone of smooth muscle, and to increase the resistance of the body to bacterial disease.

In all our work we find the results of a deficiency of either vitamin C or vitamin D, together with an excess of the other vitamin, are affected by the amount of calcium in the diet. There is a marked delay in even the partial healing of ulcers with a minimal amount of vitamin C when there is a deficiency of calcium in the diet, and the body is less resistant to the spread of bacterial disease. The lowered resistance to bacterial disease is also exaggerated by a decided excess of calcium, which may act by reducing the phagocytic activity of the blood. The following experiments were made to determine the influence of the vitamin C, vitamin D, calcium balance on infection of guinea-pigs by organisms from the intestine.

[9] Studies in Deficiency Diseases, 1922, p. 95.

[10] Grant, A. H., and Isaacs, M. L.: Cincinnati M. Bull., 1924, 31, p. 52. Grant, A. H.: Pending publication.

[11] Grant, A. H.: Pending publication.

EXPERIMENTAL WORK

The first series was planned to repeat, with a controlled amount of B. aertrycke, observations that had been worked out in a preliminary study. Here we found that an excess of cod liver oil (3 cc. per day) with a scorbutic diet decreased the resistance of the intestinal walls until the aertrycke organism migrated through, and into the blood stream in almost 50% of the cases. The addition of even small amounts of vitamin C (1.5 cc. orange juice) prevented this migration of the bacillus. The substitution of olive oil for the cod liver oil in the vitamin C

TABLE 1

RELATION OF DIET, with EXCESS VITAMIN D AND DEFICIENT VITAMIN C, TO B. AERTRYCKE INFECTIONS IN UNINOCULATED GUINEA-PIGS

Supplements to Basal Diet	Amount, Cc.	Number of Guinea-Pigs	
		Used	Infected
	...	20	0
Cod liver oil	3.0	20	10
Cod liver oil Orange juice	3.0 1.5	12	0
Calcium lactate *	1.0	10	1
Calcium lactate with:			
Cod liver oil	1.5	3	1
Cod liver oil	3.0	16	12
Cod liver oil Orange juice	3.0 1.5	3	1
Cod liver oil Orange juice	3.0 9.0	2	1†
Oxidized cod liver oil	3.0	3	3
Olive oil	3.0	4	0

* 1 gm. calcium lactate per 100 gm. oats in basal diet.
† Very slight infection in right middle lobe (lung).

deficient diets did not decrease the resistance of the intestines below that found when scorbutic diets alone were given. The basal diet consisted of rolled oats, with 1% NaCl, and timothy hay.

We found that an excess of calcium (1 gm. calcium lactate per 100 gm. oats) in the diet favored the migration of bacteria into the blood-stream, and into the lungs, liver and spleen. With an excess of both calcium and cod liver oil, and a deficiency of vitamin C, the aertrycke organism migrated through the intestinal wall, and into the blood stream in 75% of the cases. The addition of small amounts of vitamin C (1.5 cc. orange juice) lessened the percentage of infected cases, but even with 9 cc. amounts there was some migration. Oxidizing the cod

liver oil destroyed its content of vitamin A, but it did not prevent the sharp decrease in intestinal resistance caused by an excess of vitamin D.

For the series inoculated with 5.0 cc. of a culture of B. aertrycke by mouth, the guinea-pigs were taken from stock on a diet of timothy hay, rolled oats (with 1% NaCl) and cabbage, and were given the experimental diets when they were inoculated.

We found that the intestines were less resistant to a small dose (0.5 cc.) of a pure culture of B. aertrycke than they were to the number of bacilli that were normal to the intestines; but that the relative

TABLE 2

RESULTS WITH DIFFERENT DIETS IN GUINEA-PIGS INOCULATED BY MOUTH WITH 0.5 CC. B. AERTRYCKE CULTURE

Length of experiment—32 days

Supplements to Normal Diet	Amount, Cc.	Number Guinea-Pigs		Infection			
		Used	Killed	Liver	Spleen	Lungs	Blood
Normal diet (hay, oats [1% NaCl] and cabbage)..	...	2	2	0	0	0	0
Cod liver oil	3	6	3	5	6	5	3*
Cod liver oil	3						
Orange juice	9	4	3	0	0	0	0
Cod liver oil	3						
Orange (vitamin destroyed)	9	2	1	1	2	1	1*
Cod liver oil	3						
Orange (alkaline)	9	2	1	1	2	1	1*
Olive oil	3	3	2	0	0	0	0
Olive oil with cholesterol	3	1	0	0	0	0	0
Scorbutic diet without cabbage	...	2	1	1	2	0	1*
Controls—uninoculated guinea-pigs							
Cod liver oil	3	3	3	0	1	1	0
Olive oil	3	2	2	0	0	0	0
Olive oil with cholesterol	3	4	4	0	0	0	0
Scorbutic diet without cabbage	...	4	2	0	0	0	0

* Cultures of heart blood taken only when guinea-pig was killed.

resistance followed the same laws as for the milder infection. With a deficiency of both vitamin C and vitamin D the resistance of the intestines was very low; with a normal diet the pigs were immune; and with an excess of vitamin D (3 cc. of cod liver oil per animal daily) the intestinal walls were readily permeable even when cabbage was given to furnish enough vitamin C for maintenance. The substitution of olive oil, or olive oil and cholesterol, for the cod liver oil prevented this migration of the bacilli through the intestinal wall.

The addition of large amounts of orange (9 cc. per day) prevented the decrease in resistance due to an excess of vitamin D. Destroying vitamin C, by making the orange alkaline with $NaHCO_3$ and boiling,

permitted the migration of the bacillus through the intestinal wall; but as the addition of $NaHCO_3$ to fresh orange juice did the same thing the decreased resistance could not be attributed to the destruction of vitamin C.

In the group that was not inoculated the vitamin C of the cabbage almost prevented the penetration of the intestinal wall by bacteria which occured when an excess of cod liver was given with a scorbutic diet (table 1). On this diet the lungs were slightly infected in one pig, and in one the spleen was roughened. Cultures of these organs contained small colonies of the bacillus. The substitution of olive oil, or olive oil and cholesterol, for the excess of cod liver oil did not lower the resistance of the intestines below normal.

In a second series inoculated by mouth with 2 cc. of a culture of B. aertrycke the course of the bacillus in the body was studied with different diets. This large dose enabled us to detect the comparatively slight defects of the diet, such as the defects caused by an excess of vitamin C, together with a deficiency, or comparative deficiency of vitamin D. Many of the diets were begun two weeks before inoculation, so that lowering the resistance of the intestines both before and after inoculation could be studied. The amount of culture given was so large that it proved to be toxic for all the animals; a few died from shock, several died within a week, almost all lost in weight, following ingestion, and the resistance of every one was lowered. When the diets were begun 2 weeks before inoculation, every guinea-pig, except those on the very best diets, developed the characteristic nodules of the B. aertrycke in either the spleen or liver; but even here the effects of the diet could be traced through the entire series. With this very large dose the spleen was the first organ to be infected, and, in the more severely unbalanced diets, the nodules were numerous, and very large. The liver was infected in less than half of the guinea-pigs, and the lungs in two.

The addition of an excess of calcium was either not beneficial, or was distinctly harmful. With a deficiency of vitamin D, and a partial deficiency of vitamin C, the injurious effects were pronounced. Although the two pigs on this diet died 3 and 7 days after inoculation both were badly infected. Without the excess of calcium the infection was very slight in a pig killed 25 days after inoculation. The addition of a large amount of orange juice (9 cc. daily) to a diet containing an excess of cod liver oil, without an excess of calcium, entirely protected the pigs. That this protection was due to the addition of vitamin C was plainly shown when the oxidation of the orange, with boiling, resulted in a

severe infection, both with and without an excess of calcium. The addition of an excess of orange to a diet deficient in vitamin D resulted in a severe infection, both with and without an excess of calcium. The addition of an excess of orange to a cabbage diet resulted in an infection of a milder type. When scorbutic diets were given before inoculation resistance was not improved very much by a cabbage diet given after inoculation. The guinea-pigs on this diet were badly infected, although the pig of the same size that was given the cabbage from the beginning was immune. The addition of either $NaHCO_3$ or citric acid to a cabbage diet did not lower the resistance below that of very young pigs on the cabbage diet. As very young pigs were used for this diet they were compared with a very young pig on the cabbage diet that was slightly infected. The large dose used for such very small pigs easily accounted for the small amount of infection that was found on these diets. Taking the guinea-pigs from stock and beginning the experimental diets on the date of inoculation gave the same results as starting the diets two weeks before inoculation. A well balanced diet given before inoculation did not protect the guinea-pigs from a poorly balanced diet given after that date, any more than a well balanced diet given after inoculation protected from a deficiency before that date. In both cases the pigs were severely infected.

CONCLUSIONS

An adequate balance between dietary calcium, vitamin C and vitamin D is essential in maintaining the normal resistance of the intestines to the migration of intestinal bacteria into the blood stream.

A severe disturbance in the proper ratio between vitamin C and vitamin D increases the permeability of the intestines to bacteria. This is true both when there is an excess of vitamin D and a deficiency of vitamin C, or an excess of vitamin C and a deficiency of vitamin D.

The oxidation of cod liver oil destroys its content of vitamin A, but it does not destroy the factor that is responsible for the marked decrease in intestinal resistance.

Oxidizing the orange juice destroys its power to inhibit the factor in cod liver oil that is responsible for the marked decrease in intestinal resistance due to the cod liver oil factor.

An excess of calcium seems to favor the migration of bacteria into the blood stream whenever used; and when vitamin C and vitamin D are both deficient, it seems to favor a rapid multiplication of the bacteria in the spleen and lungs.

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